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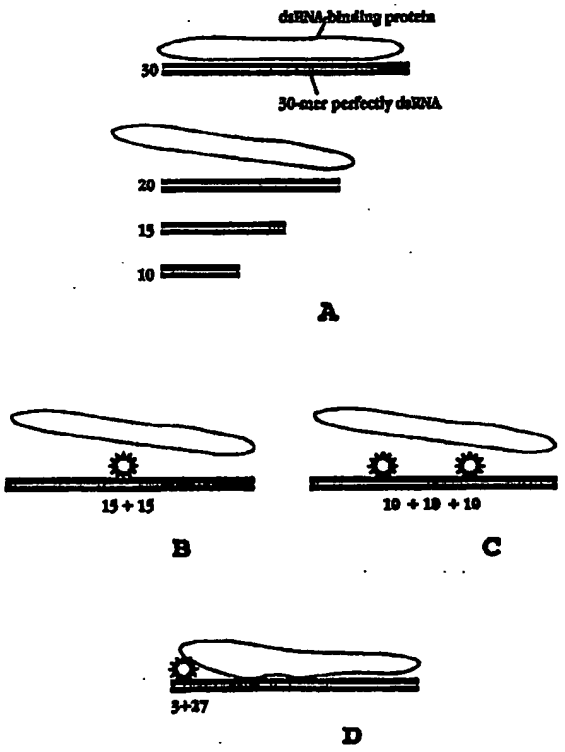
(54) Title: dsRNA/dsRNA–BINDING PROTEIN METHODS AND COMPOSITIONS

(57) Abstract

Disclosed is a screening method for identifying compounds capable of disrupting binding of a dsRNA–binding protein or protein complex to dsRNA. Also disclosed are methods of treating viral infections using such compounds.



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dsRNA/dsRNA-BINDING PROTEIN METHODS AND COMPOSITIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/058,740, filed on September 12, 1997, incorporated herein by reference in its entirety.

Portions of this work were funded by the Defense Advanced Research Projects Administration (grant no. N65236-98-1-5400). Accordingly, the United States Government may own certain rights in this invention.

Field of the Invention

The invention relates to a screening method for identifying compounds capable of binding to duplex dsRNA, and particularly, of disrupting binding of a dsRNA-binding protein or protein complex to dsRNA. The invention also relates to concatemeric compositions of compounds identified by such screening, and to methods of treating disorders and diseases such as viral or virioid infection using such compounds.

Background of the Invention

A number of approaches have been proposed and implemented to regulate gene expression, including regulation at the level of transcription of DNA as well as translation of polypeptides. For example, anti-sense molecules may be targeted at the mRNA in organisms which utilize the DNA-directed mRNA synthesis intermediates.

While such anti-sense and protein-based technologies can be used to regulate gene expression *in vitro*, small organic molecules are generally considered more desirable for drug development. Such molecules are more likely to cross cell membranes, and effect biological responses *in vivo*. Further, since each new mRNA and protein target has a unique folded structure, rather intensive drug discovery efforts are normally

required to identify drugs directed to specific sequences.

Regulation of gene expression has particular utility in the area of control of viral and virioid infections. Although they often use components of the host cell machinery for replication, the genetic material of many viruses and virioids is different from that of the host cell and is therefore a potential target for therapeutic control.

For example, RNA viruses are distinguished by having single-stranded or double-stranded RNA as their primary genomic materials. However, during replication, RNA viruses must pass through a stage in which duplex double-stranded RNA (duplex dsRNA) is formed. Duplex dsRNA is distinguishable from RNA found in hosts, by virtue of its content of canonical dsRNA having uninterrupted Watson-Crick base-pairing, as discussed below. In addition, several RNA (and DNA) viruses have long, well-conserved stem-loop structures, in which the stem is uninterrupted dsRNA. In contrast, host organisms lack long stretches of duplex dsRNA; instead they have only *very short* stretches (generally less than about 6-10 base pairs) of defect-free double-stranded RNA, such as is formed from the self-folding of single-strand transcribed RNAs (Doudna, J.A. Nature 388: 830-831, 1997).

Interferons (IFNs) are a family of cytokines that are produced by the cells of vertebrate animals in response to viral infections and other cellular stresses. Binding of INF to the receptors on the surface of INF responsive cells triggers a signaling cascade, which results in induction of more than 30 IFN-inducible proteins. (Sen G. C. and Ranshoff R. M., Adv. Virus Res. 42: 57-63, 1993; Jaramillo M.L., *et al.*, Cancer Invest., 13: 327-338, 1995). Among the interferon induced proteins is dsRNA-activated protein kinase (PKR). Activation of PKR by long dsRNA is an

important element of interferon-induced cellular immune response, which is a first line of defense against viral infection within the animal host. The activation of PKR appears to inhibit viral protein synthesis. PKR appears to bind dsRNA and phosphorylates the α-subunit of the eIF-2 transcription factor. Posphorylated eIF-2 is inactive and protein synthesis in the virus infected cells is shut down (Katze, M. G. Semin. Virol:4: 259-268, 1993).

There are ways in which viruses neutralize PKR activation by dsRNA. This is indicative of the ubiquitous presence of dsRNA in virus-infected cells, a characteristic observed for a wide variety of viruses and the central role that PKR plays in cellular response to viral injection.

For example, Adenovirus and Epstein-Barr virus produce RNA molecules, which are competitive inhibitors of PKR-dsRNA binding reaction. Similarly, vaccinia virus protein, K3L, and hepatitis C virus protein NS5A block PKR-dsRNA interaction acting as pseudosubstrates. Influenza, vaccinia and reoviruses produce NS1, E3L, and sigma 3 proteins, respectively, which bind to dsRNA. Influenza virus also induces the production of a cellular protein p58, which is a natural inhibitor of PKR. Poliovirus infection leads to PKR proteolysis. Some viruses developed multiple mechanisms to disrupt PKR activation by dsRNA. For example, HIV Tat protein lowers expression of PKR. HIV TAR RNA binding protein binds to PKR and inhibits its kinase activity, and is a competitive inhibitor of dsRNA binding (Gale, M., *et al.*, Pharmacol. Ther. 78: 29-46, 1998).

Clearly, in the course of viral evolution, viruses have developed various effective mechanisms of disrupting interferon-induced activation of PKR by dsRNA. Because of these mechanisms, interferon is not a very effective treatment of viral infection. However, interferon in treatment of viral infection is a broad

spectrum anti-viral drug, as it recognizes dsRNA, an intrinsic feature of replication of many viruses. There is a need to develop broad spectrum drugs which can recognize long dsRNA and inhibit virus replication.

Summary of the Invention

The relative lack of longer duplex dsRNA target regions in host cells make it unlikely that compounds targeted at *long* stretches of dsRNA will exert any mechanism-based toxicities in mammalian cells. Such compounds are therefore candidates for useful therapeutics.

The present invention includes a duplex dsRNA:protein binding assay that is useful for screening libraries of chemically or biologically derived synthetic or natural product compounds. Compounds are tested for their abilities to alter base pair sequence-independent binding of dsRNA binding proteins to a dsRNA test sequence. The compounds can inhibit or promote binding of dsRNA binding proteins to dsRNA. This assay is used to screen compounds, which can then be used to construct new, more potent binding agents and/or agents that deliver "warheads" effective to modify and thereby inactivate the target duplex dsRNA. Such agents may thereby interfere with replication of dsRNA by hindering strand separation, disrupting binding of essential proteins to the RNA or by inactivating the dsRNA.

Apart from replicative intermediates, long dsRNA may also be produced during the transcription of genomes of several groups of RNA viruses, especially segmented and non-segmented negative strand RNA viruses and ambisense RNA viruses (Roizman B. & Palese, P. Multiplication of viruses: an overview. *In*: Fields Virology, v1 Lippincott-Raven publishers, Philadelphia, New York, 1996, pp. 101-111). Production of mRNA from negative strand virus RNA template results in long stretches of dsRNA. Therefore, dsRNA present in cells

infected with negative strand and ambisense RNA viruses are also useful in the practice of this invention.

Double-stranded binding agents selected using the methods of the present invention are particularly useful as therapeutics for viral and virioid infections, since these infective agents utilize relatively long dsRNA molecules during its lifecycle. The assay of the invention is particularly useful in the discovery and development of small-molecule drugs which recognize dsRNA and interfere with propagation of RNA viruses with minimal interference with the host cell functions. However, the methods and compositions are not limited to this purpose. Mammalian and other eukaryotic cells, as well as prokaryotic cells, also include certain dsRNA regions which may also serve as targets for compounds discovered and constructed in accordance with the invention.

The present invention relates, in general, to methods for screening for and selecting compounds that affect base pair sequence-independent binding of dsRNA-binding proteins or protein complexes to dsRNA. In one embodiment, the method is directed to screening compounds that disrupt such binding. In a more general embodiment, the invention includes methods for screening compounds that affect dsRNA function, such as dsRNA segments involved in cellular function. In yet another embodiment, the compounds identified by this invention are useful in detecting infection in animals and other organisms. As described below, more particular embodiments of the invention are related to discovery and construction of compounds that are targeted to RNA-viruses and virioids.

According to one embodiment of the invention, the screening method includes the steps of (a) forming a reaction mixture composed of (i) a dsRNA-binding protein or protein complex, and (ii) a dsRNA to which the binding protein can bind, independent of dsRNA base pair

sequence; (b) detecting the level of binding of dsRNA-binding protein to the dsRNA fragment in the presence and absence of a test compound based on the difference in binding observed in the presence and absence of the compound being greater or lower than a selected value; and (c) selecting the test compound as capable of altering the binding if the level of binding detected is substantially different from that in a control reaction mixture (in the absence of test compound). In a related embodiment, the method is useful for identifying compounds that selectively bind dsRNA, that is, compounds that exhibit at least a 2-3-fold higher affinity for binding dsRNA than for single-stranded RNA (ssRNA), single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA).

In one embodiment, the concentration of dsRNA-binding protein or protein complex present in the assay is in an amount sufficient to achieve fractional saturation of at least 25%, meaning that at least 25% of the dsRNA in the reaction mixture is complexed with the dsRNA-binding protein, in the absence of the test compound. Alternatively, the amount of the RNA-binding protein present is in an amount sufficient to achieve fractional saturation of at least 50% or 75%. Preferably, the concentration of the RNA-binding protein is present in an amount sufficient to achieve fractional saturation of at least 90%. Preferably, compounds of this invention bind to dsRNA in a sequence non-specific manner. Alternatively, the compounds may bind to dsRNA in a sequence-specific manner.

The method may further include testing the toxicity to mammalian cells, of any compound selected as above, as a further screen for useful therapeutics.

In a preferred embodiment, the dsRNA fragment used in the assay is between about 10-100 base pairs in length, more preferably about 16-50 base pairs in length and still more preferably 30-50 base pairs in length.

In still other preferred embodiments, the method is used for identifying anti-viral compounds. According to this embodiment of the invention, the dsRNA binding protein used in the assay is of viral origin, such as E3L or sigma3 protein. The dsRNA binding domains of such proteins may also be used. Such proteins (or domains thereof) can be produced recombinantly, according to methods known in the art.

According to further embodiments of the invention, the dsRNA binding protein may also be a cellular protein such as PKR, DRAF1, DRAF2, RNAaseH, Z-DNA binding protein, La (SS-B), TRBP, and dsRNA-specific adenosine deaminase.

The level of binding in the assay can be detected by measuring directly the formation of the binding complex (protein:dsRNA), or by measuring downstream or ancillary events, such as phosphorylation of one or more substrates in a cascade of reactions resulting from the dsRNA binding event. Examples of direct detection include (i) a gel band-shift assay, (ii) a scintillation proximity assay, and (iii) a filter binding assay.

An example of measuring downstream events includes the dsRNA-dependent protein kinase cascade, where, for example, auto-phosphorylation of the interferon-induced protein kinase referred to as PKR or phosphorylation of eIF-2 and/or another phosphorylatable indicator protein occur when the proper reagents, including ATP, are present in the reaction mixture. Alternatively, the specific activity of the dsRNA binding protein can be measured, where such activity is stimulated or reduced in response to binding. An example of this is the aforementioned protein kinase-mediated autophosphorylation of PKR or of its substrate eIF-2.

In a related embodiment, the invention includes a method of treating subjects or cells infected with an RNA virus. According to this aspect of the invention, a compound selected or formed in accordance with the

screening assay of the present invention is administered to the subject in an amount effective to inhibit such dsRNA binding protein to said dsRNA region present in the subject's cells. The method may include identifying in the genome of the virus a specific dsRNA base pair sequence to which a compound can be targeted and for which it can be selected, in accordance with a form of the assay that selects for base pair sequence specific compounds, discussed herein. However, such sequence specificity is not required, since, in its more general form, the assay of the invention selects compounds that recognize duplex dsRNA in a sequence non-specific manner. Here, without committing to any particular mechanistic theory, it is thought that the compounds may recognize and bind to the duplex dsRNA structure. Compounds selected on this basis are particularly useful in combating viral infections of unknown etiology and/or composition.

Another aspect of this invention includes a method of detecting the presence of a viral agent in a biological sample. dsRNA binding compounds identified by the methods disclosed herein can be used to determine if a biological sample contains viral agents. In this embodiment, viral or virioid agents isolated from biological samples are tested to determine if the viral agents bind to compounds identified by these methods. Because these compounds may bind to dsRNA in a sequence-independent manner, the presence of unidentified viral agents in a biological sample can be determined.

Included in the present application is a partial list of viral and virioid pathogens for which drugs selected in the assay can be tested and used. In yet another embodiment, the compound undergoes cellular toxicity testing before it is tested for use in the organism, such as a human.

The invention further includes a method of screening for a compound that is capable of base-

specific binding to a 3-16, a 3-8, or more specifically, a 3-5 base pair region in a duplex dsRNA. The method includes: (a) forming a reaction mixture, containing (i) a test compound, (ii) a dsRNA-binding protein or protein complex, and (iii) a duplex dsRNA fragment which includes a region to which the binding protein binds in the absence of test compound, wherein the region contains at least one test sequence consisting of 3 to about 16 (alternatively 3-8) base pairs; (b) detecting the level of binding of the dsRNA-binding protein to the dsRNA fragment in the mixture; and (c) selecting the test compound as capable of binding to the dsRNA sequence if the mixture exhibits a substantially lower level of binding of the binding protein to the dsRNA in the mixture than is measured in a reaction mixture in the absence of the test compound.

In one embodiment, the assay described above can be practiced to identify compound-specific binding to a sequence in a dsRNA. Here, a compound is added to duplex dsRNA fragments containing, preferably in equimolar amounts, multiple combinatorial 3-16 (or 3-8) base pair sequences incorporated into the fragments. After binding, bound and unbound dsRNA are separated, such as by a preparative bandshift mobility gel assay, and the sequences of the unbound dsRNA fragments can be identified using amplification techniques, such as PCR, preferably in an iterative format, as described herein.

The compounds identified by this method can be used to detect or treat specific RNA viruses containing the identified compound-specific sequence.

In a related aspect, the invention also includes multimeric RNA binding agents which bind selectively to dsRNA. Such agents are linear concatemers formed by covalently linking at least two dsRNA-binding compounds, each of which is selected in accordance with the assay of the invention. Preferably neither of the compounds is an oligonucleotide or a peptide; however, they may be

linked together by any appropriate linker, including, but not limited to, a polysaccharide, a peptide or an oligonucleotide. According to a preferred embodiment, the composition includes a dsRNA-modifying agent which is effective to modify or destroy the dsRNA function.

In still other related embodiments, the invention includes methods of ranking the ability of selected compounds to bind to duplex dsDNA, as well as diagnostic kits that employ the dsRNA selection and identification methods, and/or the compositions, described herein.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Figures

FIG. 1 shows a schematic diagram of the cloning and construction of a plasmid vector for expression of dsRNA binding domains (dsRBDs) of Staufen, E3L, mPKR and hPKR proteins as fusion proteins with GST;

FIG. 2 shows a computer-generated image of autoradiographs of gels illustrating binding of E3L to radiolabeled 30-mer dsRNA;

FIGS. 3A and 3B show computer-generated images of autoradiographs of gels showing binding of E3L to dsRNA, ssRNA and RNA/DNA hybrid molecules;

FIG. 4 shows a computer-generated image of an autoradiograph of a gel showing E3L binding 19-, 22-, 26- and 30-mer dsRNA fragments, where E3L was present at concentrations of 67 nM, 12 nM and 2 nM, as indicated;

FIGS. 5A-5D show schematic representations of binding of a dsRNA-binding protein to dsRNA fragments;

FIGS. 6A-6B show computer-generated images of autoradiographs of gels showing competition by various concentrations of ethidium bromide (EtBr) for E3L binding to dsRNA (30mer) on a plain gel (A) and a gel

containing 3.3μM EtBr;

FIGS. 7A-7G show schematics of various dsRNA constructs in accordance with the invention, including a 34-mer which displays all possible permutations of trimeric structures as SEQ ID NO: 1 (7A);

FIG. 8 shows a schematic of the dsRNA-dependent protein kinase PKR cascade of cellular events;

FIG. 9(A-C) shows a schematic of the scintillation proximity assay using a streptavidin-SPA bead and a biotin labeled dsRNA;

FIG. 10 shows ten oligonucleotides (30-mer) which together provide all 256 possible combinations of oligonucleotides of four nucleotides in length. The ten oligomers were derived from one contiguous sequence which include the 256 possible combinations;

FIG. 11 shows the ^{33}P end-labeling, biotinylation, and annealing to a complementary oligonucleoide of one of the oligonucleotides shown in FIG. 10;

FIG. 12 shows the results of an SPA biotin protection experiment using Staufen, E3L, mPKR and hPKR as the RNA binding proteins. These results are described in Example 6;

FIG. 13 shows the results of the SPA using hPKR and the indicated RNA binding compounds. The results are described in Example 6;

FIG. 14 shows the results of screening novel compounds from a combinatorial chemistry library. Fig. 14A shows the results of screening 44 series 1a compounds (compounds A2-12, B2-12, C2-12, and D2-12). Fig. 14B shows the results of screening 54 series 1b compounds (compounds A2-12, B2-12, C2-12, D2-12, and E2-11). The results are described in Example 7; and,

FIG. 15 shows the results of fluorescence/quench assays performed to determine if compound B5 (plate 1a) preferentially binds to the indicated polynucleotides as described in Example 8.

Detailed Description of the Invention

I. Definitions

A "small molecule", as the term is used herein, refers to a biologically or chemically synthesized organic or inorganic compound that is generally less than about 10,000 molecular weight, and most commonly less than 1,000 molecular weight. A small molecule is preferably permeable to cells, and is not usually a polypeptide or a polynucleotide.

A "polypeptide" as used herein refers to a polymer of the 20 naturally occurring amino acids.

A "polynucleotide" as used herein refers to a polymer of the five naturally occurring nucleotides of adenosine, cytosine, guanosine, thymidine and uracil.

A "concatemer" as used herein refers to a linear assembly of small molecules to form a multimeric small molecule. A concatemer useful in this invention may include small molecules covalently linked through one or more polyamide linkages.

"Duplex dsRNA" as used herein refers to a long stretch (at least 10 base pairs) of canonical dsRNA that exhibits Watson-Crick base-pairing all along its length with no mismatches, loops, bulging, internal base pairing, or the like. Duplex dsRNA is exemplified by the genomes of many dsRNA-viruses. However, the binding sites of a small molecule, whether sequence non-specific or sequence-specific, may span 3-6 base pairs, 7-10 base pairs, or 10-20 base pairs.

An "RNA modifying agent or moiety" refers to a compound which, when it contacts RNA, effects RNA cleavage, or otherwise modifies it or renders it non-functional. Examples include, but are not limited to alkylating agents, such as nitrogen mustard reagents, pro-reactive cyclopropyl functionalized polyheteroaromatic reagents, psoralens, Lewis acid metal chelate complexes, ene-dynes, and activated higher valency metallo-oxo complexes.

The term "adjacent" as used in reference to areas in a dsRNA molecule, refers to regions that are separated by as few as zero, but no more than about 20, base pairs.

The term "duplex dsRNA base pair specific binding" refers to compounds that bind to a specific sequence of ribonucleotides in a dsRNA. In contrast, the terms "duplex dsRNA base pair non-specific binding" and "duplex dsRNA structural binding" refer to binding to duplex dsRNA that is not dependent on the presence of a particular sequence within the RNA, but rather appears to be dependent on the presence of characteristic secondary or tertiary structure.

The term "duplex dsRNA selective binding" refers to binding between a molecule and duplex dsRNA with an affinity that is higher (preferably at least about 2-3-fold higher) than binding of the molecule to either dsDNA, ssDNA, or ssRNA. Affinity measurements or approximations can be made according to methods well known in the art, and exemplified in Example 3 herein.

"Binding," as used herein, usually refer to the noncovalent association of a protein or small molecule with an RNA molecule. Functional binding of the protein to dsRNA is measured using one or more of a number of known binding assays, including, but not limited to, gel mobility shift assay.

"On-rate" is herein defined as the amount of multimeric complex formed from its monomeric precursors in a given unit of time.

"Equilibration time" is herein defined as the time required for two molecules to reach a steady-state of association and dissociation: for example, the RNA:protein complex.

"Off-rate" is herein defined as the amount of multimeric complex that dissociates in a given unit of time.

"Dissociation" is the process by which two (or more) molecules cease to interact or bind to one another: the process usually occurs at a fixed average rate under specified conditions.

"Half-life" is herein defined as the time required for one-half of the associated complexes, e.g., RNA:protein complexes, to dissociate.

"Fractional saturating amount" as used herein is defined as the concentration at which the RNA binding protein or protein complex binds to at least a certain defined fraction of available dsRNA. To achieve fractional saturation of 50%, the required concentration of RNA binding protein is the thermodynamic binding constant K_d if the concentration of RNA is significantly lower than the value of K_d. To achieve fractional saturation of 90%, the required concentration is at least 10-fold greater than K_d. Thus, for an RNA-binding protein that has a k_d of $1x10^{-9}$ M, to achieve fractional saturation of 90%, the amount of the RNA-binding protein required is at least $10x10^{-9}$ M.

"Monomer subunit molecules (or compounds)" are small molecules that bind to sites on double-stranded RNA spanning about 3-16 base pairs. They may or may not exhibit sequence-specific binding.

"Dimer molecules (or compounds)" are small molecules that consist of two covalently chemically linked monomer subunit molecules. Such molecules bind to a larger binding site than the monomers from which they are made and usually, but not necessarily, bind to the dsRNA with a higher affinity.

"Sequence-specific binding" refers to RNA binding molecules that exhibit a strong RNA sequence preference to specific base pair sequences present in fully double-stranded RNA.

"Test sequence" is the RNA sequence that defines or is allowed at the cognate binding site for an RNA binding protein. In the context of the present

invention, a test sequence is preferably fully embedded within the test duplex dsRNA fragment.

The terms "polymerase chain reaction" and "PCR" refer to a process of amplifying one or more specific nucleic acid sequences, wherein (i) oligonucleotide primers which determine the ends of the sequences to be amplified are annealed to single-stranded nucleic acids in a test sample, (ii) a nucleic acid polymerase extends the 3' ends of the annealed primers to create a nucleic acid strand complementary in sequence to the nucleic acid to which the primers were annealed, (iii) the resulting double-stranded nucleic acid is denatured to yield two single-stranded nucleic acids, and (iv) the processes of primer annealing, primer extension, and product denaturation are repeated enough times to generate easily identified and measured amounts of the sequences defined by the primers. The sequential annealing, extension and denaturation steps are controlled by varying the temperature of the reaction container, normally in a repeating cyclical manner. Annealing and extension are typically carried out between 40-80°C, whereas denaturation requires temperatures between about 80 and 100°C. A "thermal cycler", such as Perkin Elmer Model 9600, is typically used to regulate the reactions.

The term "polynucleotide" as used herein refers to a polymeric molecule having a backbone that supports nucleic acid bases capable of hydrogen bonding to typical polynucleotides, where the polymer backbone presents the bases in a manner to permit such hydrogen bonding in a sequence specific fashion between the polymeric molecule and a typical polynucleotide. Such bases are typically inosine, adenosine, guanosine, cytosine, uracil and thymidine. Polymeric molecules include double and single stranded ribonucleic acids (RNA) and deoxyribonucleic acids (DNA), and may include

polymers having backbone modifications such as methylphosphonate linkages.

The term "vector" refers to a nucleotide sequence that can assimilate new nucleic acids, and propagate those new sequences in an appropriate host. Vectors include, but are not limited to recombinant plasmids and viruses. The vector (*e.g.*, plasmid or recombinant virus) comprising the nucleic acid of the invention can be in a carrier, for example, a plasmid complexed to a protein, a plasmid complexed with lipid-based nucleic acid transduction systems, or other non-viral carrier systems.

The term "polypeptide" as used herein refers to a compound made up of a single chain of the twenty natural amino acid residues linked by peptide bonds. Amino acid residues are referred to herein by their standard single letter notations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, Isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. The term "protein" as used herein may be synonymous with the term "polypeptide" or may refer, in addition, to a complex of two or more polypeptides. In the context of the present invention, a "protein complex" refers to complexes of protein or proteins that bind to a single ribonucleotide fragment. The complexes may be homomeric or heteromeric. Generally, but not always, polypeptides and proteins are formed predominantly of naturally occurring amino acids.

Nucleic acid subunits are referred to herein by their standard base designations; T, thymine; A, adenosine; C, cytosine; G, guanine, U, uracil; variable positions are referred to by standard IUPAC abbreviations: W, A or T/U; R, A or G; S, C or G; K: G or T/U (37 CFR §1.822).

"Base pair sequence-independent binding" refers to binding between a dsRNA-binding protein or protein complex and dsRNA whose binding affinity is independent or substantially independent of dsRNA sequence.

II. dsRNA:dsRNA-binding protein Displacement Assay

A. Overview of Assay

The assay of the invention is directed to detecting compounds that are capable of disrupting binding between double-stranded RNA (dsRNA) and binding proteins which specifically bind to such dsRNA. In this context, the term "specifically binds" means that such binding proteins preferentially bind to dsRNA relative to binding of ssRNA, ssDNA, dsDNA, or RNA/DNA hybrids. The assay is also adapted to detect sequence preferential and/or sequence specific binding by compounds to particular RNA sequences within the dsRNA test binding nucleotide.

The assay can be used to detect compounds for a variety of therapeutic uses. Foremost among these uses is use in identifying compounds for use as anti-viral agents, however, since the assay particularly selects for disruption of binding to duplex dsRNA -- dsRNA of greater than about 30 nucleotides in length which are coiled in a double-helix configuration. That is, it is known that many invading viral pathogens contain long stretches of complementary double stranded RNA during some or all of their life cycles, whereas in most eukaryotic cells, including mammalian cells, the self-folding of the single strand of transcribed RNAs rarely creates stretches longer than a few base-pairs of double strand sequences lacking any defects (i.e., mismatches, bulges and internal loops). Thus the assay is particularly useful in selecting for anti-viral agents which have minimal effects on the cellular machinery of host cells.

To carry out the assay method of the invention, generally, test compounds are tested for ability to alter binding of a selected dsRNA-binding protein or protein complex from a fragment of dsRNA to which the binding protein binds or alter the affinity of this binding interaction. The compound can inhibit or enhance the binding of the dsRNA-binding proteins or protein complex to the dsRNA fragment. Binding levels in the presence of the compound are compared to levels measured in the absence of test compound, and the compound is selected if the binding level in the presence of compound is greater than a selected threshold, e.g., only one-half or one-third.

These features of the invention are described in detail below and in Example 3 herein. The sections that follow describe the design and selection of the various components that form the basic assay mixture.

B. Components of Assay

The basic binding assay includes a reaction mixture that is composed of (i) a dsRNA binding protein, (ii) a fragment of dsRNA to which the binding protein binds, in a base pair sequence-independent manner, and (iii) the test compound. The reaction is monitored for differential levels of binding of dsRNA binding protein in the absence and in the presence of the test compound.

1. dsRNA-Binding Proteins

While a number of dsRNA binding proteins have been isolated and characterized from cellular and viral sources, research on these proteins has revealed no sequence specificity in binding of known dsRNA binding proteins, or more particularly, dsRNA binding protein domains (dsRBD) to dsRNA. Moreover, for at least one class of molecules, typified by vaccinia virus E3L and PKR, while the individual protein monomer may be rather small (spanning less than about 10-20 bp), the binding

proteins or domains tend to form cooperative binding units, so that two or more protein subunits bind to a sequence of RNA with a much higher affinity than any of the protein monomers or protein domains alone.

Experiments performed in support of the present invention demonstrated that a single protein type can be used to assay virtually any RNA sequence by placing the sequence of interest (test sequence) within a defined dsRNA fragment, as described below. A small molecule that specifically binds to the test sequence can be detected by alterations in the binding characteristics of the protein to the dsRNA fragment containing that sequence, in comparison to other dsRNA fragments containing different test sequences.

In choosing a dsRNA-binding protein for use in the present invention, relevant considerations include the following:

(i) The protein will preferably be specific for dsRNA binding, as opposed to ssRNA, dsDNA, or ssDNA binding, or possibly, binding to RNA/DNA hybrid.

(ii) Depending on the assay format, the half-life of the dsRNA:protein complex should be short enough to accomplish the assay in a reasonable amount of time. Drug-mediated disruption of complexes having very long dissociation times may be difficult to measure. Alternatively or in addition, the assay may be carried out in a "competitive" manner, where competition for binding, rather than displacement of binding is measured, by exposing the test compound and the binding protein to the test dsRNA fragment simultaneously, or by mixing the test molecule and the dsRNA molecule, followed shortly by addition of the binding protein.

(iii) The half-life of the complex should be long enough to allow the measurement of unbound RNA in a reasonable amount of time. For example, the level of free RNA is dictated by the ratio between the time needed to measure free RNA and the amount of free RNA

that occurs naturally due to the dissociation of the complex during the measurement time period.

In view of the above two considerations, practical useful RNA:protein half-lives fall in the range of approximately two minutes to several days: shorter half-lives may be accommodated by faster or "real-time" detection methods and longer half-lives may be accommodated by destabilizing the binding conditions for the assay.

a. Viral dsRNA-binding Proteins

Described in the art are a number of dsRNA-binding proteins that are derived from viral sources. It is appreciated, however, that compounds selected to displace binding of a particular dsRNA binding protein are not limited to use against the pathogenic source of the binding protein, but may be more widely applicable as anti-viral agents, and even cell-regulatory, agents.

By way of example, but not limitation, one dsRNA binding protein that is used in assays of the present invention is the vaccinia virus E3L dsRNA binding protein. Other dsRNA binding proteins useful in the invention are human PKR (hPKR) and murine PKR (mPKR). In experiments carried out in support of the present invention, the dsRNA binding domains of E3L, hPKR, mPKR, and Staufen were produced recombinantly, as detailed in Example 1, and purified (Example 2) prior to use as a component in a binding assay.

FIG. 1 shows a schematic diagram of the cloning and construction of a dsRNA-binding fragment of E3L, hPKR, mPKR, and Staufen. Briefly, PCR fragment which encode for the dsRNA binding domains of these proteins were obtained using forward and reverse PCR primers that were selected on the basis of the known sequence of the open reading frames (ORF) and the location of the dsRNA binding domain within the ORF (*e.g.*, Ho, C.K., *et al.*, Virology 217: 272-284, 1996). Restriction endonuclease

sites (as indicated in Fig. 1) were introduced into primers in order to facilitate cloning of the PCR fragment. The PCR fragments were ligated with a vector, as illustrated, to provide for expression under the control of an inducible promoter (*e.g.*, *tac* promoter present in the pGEX-2T vector, as shown). Following transformation of a suitable host cell line, the dsRNA binding domains were expressed as fusion proteins, which was then purified by standard methods, as detailed in Examples 2 and 6.

A number of other suitable dsRNA binding proteins or fragments thereof derived from viral sources can be used in the assay of the present invention. For use in selecting therapeutics specifically targeted to disrupting viral dsRNA-dsRNA binding protein binding, it may be advantageous, but is not necessary, to utilize dsRNA binding proteins derived from viruses.

For example, the reovirus sigma3 (σ3) protein is a major outer capsid component of the reovirus virion which also exhibits dsRNA binding activity which resides in its C-terminal dsRNA binding domain (dsRBD) which consists of about 85 amino acids from about amino acid 233 to 305 of the 365 residue sequence (Yue, Z, *et al.*, J. Virology 70: 3497-3501, 1996). Sigma3 has been demonstrated to have a stimulatory effect *in vitro* on translation of late viral mRNA (Mabrouk, *et al.*, Biochem. Cell Biol. 73: 137-145, 1995). The full length sigma3 protein can be cloned and stably expressed, for example, in a mammalian (HeLa) cell line, according to published methods (Yue, Z, *et al.*, J. Virology 70: 3497-3501, 1996, incorporated herein by reference). Modifications of this procedure, along the lines described above, can be used to produce the C-terminal region derived dsRBD, which may also be used in the assay method of the present invention.

Non-viral dsRNA binding proteins include, but are not limited to Z-DNA binding protein of 140,000 daltons

derived from chicken lung (Herbert, *et al.*, Nucleic Acids Symp. Ser. 33: 16-19, 1995); *Saccharomyces cerevisiae* RNase H1 N-terminal binding domain (Cerritelli, S.M., RNA 1: 3, 246-259, 1995); La (SS-B) autoimmune antigen which unwinds dsRNA and therefore inhibits dsRNA activated protein kinase (PKR) (Xiao, Q., *et al.*, Nucleic Acids Research, 22: 2512-2518, 1994); TRBP derived from HeLa cells which binds to the HIV-1 Rev-responsive-element RNA (Park, H., *et al.*, Proc. Natl. Acad. Sci. 91: 4713-7, 1994); dsRNA-specific adenosine deaminase from human K88 clone (two forms; one interferon inducible and the other constitutive; Patterson, *et al.*, Mol. Cell. Biol. 15(10): 5376-5388, 1995); DRAF1 and DRAF2 which are involved in activation transcription of interferon-stimulated genes in response to adenovirus or dsRNA (Daly, C., *et al.*, Mol. Cell. Biol. 13(6): 3756-3764, 1993).

b. Test dsRNA fragments

For use in the assays of the present invention, the dsRNA fragment is preferably a small duplex RNA molecule, preferably between about 10 and about 50 nucleotides in length. The optimal length of the dsRNA fragment will be determined, in large part, by the dsRNA binding protein used in the assay. Experiments carried out in support of the present invention, described below, provide guidance for optimizing the length of the dsRNA fragment used in assays of the invention.

For detecting sequence preferential or sequence specific binding of test compounds, dsRNA fragments may be constructed to include one or more variable, defined base pair sequences.

dsRNA can be produced in a number of ways. For example, the complementary strands can be individually chemically synthesized on a standard oligonucleotide synthesizer according to well-known methods, and mixed to effect annealing to form dsRNA. Alternatively a

second-strand synthetic process using a primer complementary to the priming site at the 3' end of the top-strand of the test oligonucleotide can be used to generate dsRNA. Such synthetic oligonucleotides are generally used at the pre-screen stage to differentiate between sequence-specific and sequence non-specific RNA binding molecules.

RNA molecules are also synthesized in *in vitro* transcription systems in which a hairpin oligo-ribonucleotide is synthesized from a DNA template (i.e., the linear sequence includes the top strand of the stem, the loop sequences, then the bottom complementary strand of the stem). Using this type of strategy allows for the synthesis of self-annealing dsRNA oligonucleotides with specific internal test sites, generally 3-8 bp in length. For oligonucleotides with mixed base test sites, a partial hairpin sequence can be transcribed from a DNA-template (i.e., the top strand stem, the loop, and part of the bottom strand stem), allowed to anneal and used to prime synthesis of the remaining stem structure.

Many dsRNA binding proteins have the ability to discriminate between long perfectly double-stranded RNA and shorter dsRNA or an RNA with defects in its secondary structure. For example, in experiments carried out in support of the present invention, it was found that a 30-mer dsRNA is required for efficient binding of the E3L dsRNA binding domain (dsRBD), whereas binding to shorter molecules, such as 19-mers, is detectable only at very low levels, if at all (Fig. 4).

Apparently, for hPKR binding to dsRNA, protein binds to dsRNA in a cooperative fashion, since efficiency of binding increases with the RNA size. The single dsRNA binding domain of hPKR occupies a roughly 11 base-pair site on the dsRNA, equivalent to one turn of A-form of dsRNA. However, binding to such short dsRNA could be demonstrated only at

saturating protein concentrations. Considerably better binding is observed with 16-mer dsRNA, with a significant increase in binding affinity to RNA of 22-24 base pairs in length, which can accommodate a PKR dimer. Further increase of binding is observed with longer dsRNA. 30 base pairs give even stronger binding and is a minimum size for activation of PKR with dsRNA (Manche L., *et al.*, Mol. Cell. Biol. 12:5238-5248, 1992; Schmedt C., *et al.*, J. Mol. Biol. 249: 29-44, 1995; Bevilacqua P.C. *et al.*, Biochemistry 35: 9983-9994, 1996.)

FIG. 2 shows binding of vaccinia virus E3L dsRBD to a radiolabeled 30-mer duplex dsRNA fragment having the sequence SEQ ID NO: 8 (5'to 3') in accordance with the present invention. The binding conditions are described in Example 3, herein, and binding is visualized by autoradiography of a 6% Retardation Gel. As shown, increasing amounts of E3L binding protein in the assay resulted in increased recruitment of the 30-mer dsRNA into a band having a higher apparent molecular weight, corresponding to the 30-mer-E3L complex at the top of the gel. Under the conditions used, an approximate K_d of 4-7 nM was calculated for binding. In contrast, the E3L protein did not effectively bind ssRNA (^{32}P labeled 1B) or RNA/DNA hybrid even at concentrations as high as 400 nM (FIG. 3).

Further experiments showed that the E3L protein requires a minimum size dsRNA fragment for efficient binding. FIG. 4 shows formation of E3L-dsRNA binding complexes as a function of dsRNA length (indicated at the top of the gel). From these experiments it was determined that E3L binding of a 30-mer was approximately 50-100 times more efficient than a 19-mer, and binding of the 22-mer and 26-mer was only slightly better than a 19-mer.

c. Test Compounds

Although in theory any compound can be tested in the assay of the present invention, in view of the therapeutic objective of compounds identified by the screening assay, some pharmaceutical considerations may influence the selection of a pool of molecules for use in screening. Generally preferred are small molecules less than about 10,000 molecular weight, and most commonly less than 1,000 molecular weight. The compound is preferably permeable to cells, and preferably not a polypeptide or a poly-nucleotide, which tend to be susceptible to degradation by endogenous cellular mechanisms. However, the concatemers of this invention may be linked by amide linkages (peptide bonds). In addition, small molecules are generally less likely apt to elicit an immune response, when injected into vertebrate, particularly human, subjects.

Many pharmaceutical companies have extensive libraries of small molecule compounds as either discrete or mixed entities that may be screened with the assay of the present invention. Small molecules may be either biologically or chemically synthesized organic or inorganic compounds. Synthetic libraries are available from a number of commercial sources, including but not limited to Comgenex (Princeton, NJ), Microsource (New Milford, CT), Brandon Associates (Merrimack, NH), Aldrich (Milwaukee, WI). Libraries of natural compounds derived from plants or animal extracts are available from Pan Labs (Bothell, WA) and MycoSearch (NC), for example. Such libraries can also be systematically modified to produce known or readily identifiable derivative compounds for testing. It is further understood that, in the context of the present invention, compounds selected by the screening methods described herein in a first screening can be chemically modified and further selected for enhanced dsRNA binding activity or more desirable pharmacologic properties.

Such selected, chemically modified compounds are also considered to be selected by the screening assay of the present invention.

C. Capture and Detection Systems

1. General Methods

Any of a number of capture/detection methods known in the art can be used to quantitate binding between the dsRNA binding protein and the dsRNA fragments in the binding assay of the present invention. One or both of the RNA-binding protein or the test polynucleotide fragment can be labeled, either directly and indirectly, with indicator molecules such as fluorescent dyes, radioisotopes and enzymes according to methods well known in the art. Generally, a number of appropriate methods may be found in Howard, G.C., *et al.*, *Methods in Nonradioactive Detection* (Appleton & Lange, Norwalk, CT, 1993), incorporated herein by reference.

An exemplary detection system used in experiments carried out in support of the invention is a gel mobility shift ("bandshift", "gel shift") assay, methods for which are detailed in Example 3. Here, the dsRNA fragment is end labeled with ^{32}P, and reaction products are subjected to gel electrophoresis under conditions designed to detect differences in apparent molecular weight. Gels are visualized by autoradiography, and differences in binding are evidenced by the amount of radioactive dsRNA that co-migrated with the binding protein to a position in the gel, indicating a correspondingly higher apparent molecular weight. The gels can be quantitated by commercially available gel scanners (or the Storm Phosphoimager, Molecular Devices, Mountain View, CA).

Other capture and detection methods can include direct, indirect or competitive formats and can use affinity capture systems such as streptavidin/biotin binding or immunochemical reagents, in which one or more

of the binding protein and RNA fragment, or a label or tag on either of these components, is captured. Such assays are used to detect drug-induced decreases, as well as increases, in binding between the dsRNA fragment and dsRNA binding protein present in the assay. Increases in binding may indicate a drug-induced increase in affinity between the two molecules, signifying an increased on-rate, a decreased off-rate, or both. Drug compounds which show this feature may also be useful in the context of gene regulation or drug targeting.

One exemplary capture system utilizes a "biotin protection" system. Here, a biotin molecule is linked to a base in the protein binding site in such a way that the binding of the protein is not disturbed, and when the protein binds to the site, the biotin is protected from streptavidin. Displacement of the binding protein by a drug exposes the biotin, which then binds streptavidin. This type of assay was used in a Scintillation Proximity Assay (SPA, Amersham) format to quantitate binding. Here, the radiolabeled (^{33}P) dsRNA molecule was captured by streptavidinated SPA beads impregnated with scintillant. The proximity of a radioactive isotope (e.g., ^{3}H, ^{33}P) in the oligonucleotide to the bead causes the bead to scintillate. A signal indicates the presence of an effective DNA-binding molecule; test sequences giving higher signals indicate the relative preference for different sequences. This assay was used in high throughput screening to screen for dsRNA binding compounds (Examples 7 and 8). The direct nature of the assay allows for screening complex pools of sequences, since it is possible to look for signal above a baseline.

A similar assay format can be set up such that other small, quenchable markers, such as fluorescein, are bound to the binding site.

Filter binding assays can also be adapted for use in the present invention. The set up and analysis of such assays are well known in the art.

Other suitable assays that can be used in conjunction with the assay of the present invention include, but are not limited to the following:

- indirect SPA capture assay with antibodies
- fluorescence polarization (anisotropy; Checovich, W., *et al.*, Nature 375: 254-256, 1995)
- multiplexed mass spectroscopy
- enzyme-linked immunoassay (ELISA)
- RNA PCR identification (see below)

The assay also is amenable to formatting on a bio-chip, which would greatly increase both the capacity and the complexity of sequences that could be examined. This, as well as many of the methods listed above, may be used to adapt the dsRNA-binding assay of the invention for high throughput screening.

2. RNA PCR Amplification and Identification

The above-described assay can be coupled to amplification methods (in one embodiment, polymerase chain reaction (PCR); Mullis, K.B., *et al.*, U.S. Patent No. 4,683,202; Mullis, K.B., *et al.*, U.S. Patent No. 4,683,195; Innis et al., eds. PCR Protocols, a Guide to Methods and Applications, Academic Press, Inc. (1991)) to achieve identification of RNA sequences to which binding of a test molecule is most preferred.

In this embodiment of the present invention, a dsRNA test fragment is synthesized that contains the following elements:

(i) the binding site for a dsRNA-binding protein (for example, E3L), *i.e.*, the screening site,

(ii) embedded within the screening site, at least one test sequence composed of more than two base pairs and preferably less than 20 base pairs (most preferably 3-8 bases), and

(iii) means to isolate selected sequences for amplification, such as a sufficient number of bases flanking the test site sequences to function as priming sites for polymerase chain reaction amplification or restriction sites useful to facilitate cloning.

Priming sites can also be used as primer binding sites for dideoxy sequencing reactions and may contain restriction endonuclease cleavage sites to facilitate cloning manipulations.

An example of one such a test oligonucleotide is shown in Figure 7A as SEQ ID NO: 1, which contains all possible permutations of a 3-mer. This type of polynucleotide can be subdivided to provide pooled assays. In a pooled assay format, such as can be used in conjunction with PCR elucidation of the binding sequence, as discussed below, all possible (4^3=64) trimers or (4^4=256) four base pair sequences should be represented at equimolar levels within a pool of test dsRNA fragments. From this example, it is apparent that, for an 8 base pair test sequence, all possible base pair sequences (4^8 = 65,536) would be present in equimolar amounts in the assay.

For any single-stranded test polynucleotide pool, the single-stranded molecules are annealed to a primer and the bottom strands are enzymatically synthesized by primer extension reactions. One advantage of using the assay/amplification PCR-cycling embodiment of the present invention is that it is convenient to work with larger test sequences in this embodiment. This protocol is particularly suited for determining the highest affinity binding sequences, rather than determining the rank order of binding of all test sequences; such ranking can be determined by screening individual sequences as described above.

3. Use of the dsRNA Binding Assay for Measuring Binding in Mixed Pools of Test Oligonucleotides

Using double-stranded test RNA fragments, the basic assay is performed essentially as described below (Part D): typically without the use of radioactive detection systems. Any of a number of RNA:protein combinations may be used in this assay system. One exemplary combination is the dsRNA-binding domain (dRBD) of E3L protein in conjunction with a dsRNA molecule, such as described in Example 3.

In this embodiment of the present invention, E3L dRBD is added to the test oligonucleotide pool (for example, test dsRNA polynucleotide fragment into which have been embedded 256 four base pair test sequences, which test sequences are represented at equimolar levels within the pool of oligonucleotides described above) in a reaction mixture, as described in Example 3. Candidate compounds are tested for the ability to differentially disrupt the binding of the E3L RNA:protein complex by binding to the test sequence. After the addition of a test molecule (*e.g.*, a compound derived from a combinatorial library, fermentation broth or fungal extract), the assay mixture is incubated for a desired time. The dsRNA-binding protein complexes are then separated from unbound dsRNA by any of a number of means known in the art. One convenient separation and characterization method is a preparative bandshift gel assay, in which the dsRNA polynucleotide fragments are radioactively end labeled, and the mixture is subjected to electrophoresis through a non-denaturing gel, which is then subjected to autoradiography, in order to visualize protein-bound and unbound dsRNA fragments. Alternatively, the assay mixture can be separated by chromatography, e.g., HPLC.

In one embodiment of this assay, the unbound dsRNA is amplified using polymerase chain reaction (PCR) technology. An aliquot of the resulting PCR-amplified

material is cycled through the RNA:protein binding assay again, then PCR-amplified again, in order to detect and identify sequences from which the protein is displaced during the binding assay. These isolation and reaction steps are repeated several times using each subsequent filtrate. After each PCR amplification, part of the PCR-amplified material is retained for sequencing analysis. The result of the repeated cyclings through the assay/amplification process is that the test oligonucleotide sequences that are amplified contain test sequences that are preferred binding sites for the test molecules. Through subsequent rounds of assay/amplification, these oligonucleotides are amplified to represent a larger and larger percent of the total population of amplified RNA molecules.

To summarize, the enhanced PCR strategy that is preferred in conjunction with the screening assays of the invention is an "iterative format" that includes the following steps:

1. Start with a synthetic pool of single-stranded RNA containing the sequence randomized drug testing region.

2. Copy the single-stranded RNA using an RNA primer, NTPs, and an RNA-dependent RNA polymerase activity to make the double-stranded RNA.

3. Use the binding and selection assays of the invention to separate the RNA:protein complexes from the free RNA. This may be done by a native gel-mobility shift protocol.

4. Displace the minimal detectable amount of protein bound RNA (*i.e.*, radioisotopically end-labeled) with the RNA binding test compound (*i.e.* by titrations).

5. Amplify drug-displaced RNA using PCR. Sequence the double-stranded DNA PCR product to look for consensus sequence in the test site.

6. Transcribe double-stranded DNA to make a pool of screening selected single-stranded RNA.

7. Repeat steps 1-6, if necessary to obtain sufficient material for detection.

In addition to PCR, the unbound dsRNA fraction can be amplified by other methods as well. For example, the RNA present in the filtrate can be transcribed into a corresponding DNA molecule, cloned into a selected vector (such as, phage vectors, *e.g.*, lambda-based, or standard cloning vectors, *e.g.*, pBR322- or pUC-based). The cloned sequences are then transformed into an appropriate host organism in which the selected vector can replicate (for example, bacteria or yeast). The transformed host organism is cultured with concurrent amplification of the vectors containing the cloned sequences. The vectors are then isolated by standard procedures (Maniatis, *et al.*; Sambrook, *et al.*; Ausubel, *et al.*). Typically, the cloned sequences, originally obtained from the RNA filtrate, are obtained from the vector by restriction endonuclease digestion and size-fractionation (for example, electrophoretic separation of the digestion products followed by electroelution of the cloned sequences of interest) (Ausubel, *et al.*). These isolated amplified test oligonucleotide sequences can then be recycled through subsequent rounds of assay/amplification as described above.

In another embodiment, the oligonucleotide sequences present in the original RNA filtrate can be isolated, sequenced and amplified by *in vitro* synthesis of copies of the oligonucleotides.

Sequencing of Amplified DNA. Samples from each cycle are sequenced using, for example, radiolabeled primers and dideoxy sequencing methodologies or standard chemical methodologies, using automated sequencers, according to methods well known in the art. If the amplified sequences are not sufficiently resolved to obtain unambiguous sequence information, then the resulting cDNA is further purified and sequenced.

An alternative embodiment eliminates the dsRNA amplification step. In this embodiment, the assay mixture contains the oligonucleotide pool and dsRNA binding protein. The test compounds are incubated and then separated using, for example, HPLC. Commercially available HPLC systems can provide extraordinarily high resolution. The HPLC fractions of the assay mixture can be analyzed by mass spectroscopy. The fragmentation pattern obtained from mass spectroscopy identifies the preferred binding sequences of the test compounds.

4. Measuring Modification of Downstream Factors

Another way of assessing binding takes advantage of the cascade of biological events that may be activated in cells when dsRNA is introduced to a eukaryotic host cell. FIG. 8 shows a diagram the dsRNA-activated protein kinase (PKR) cascade that is responsive to dsRNA in mammalian cells. As illustrated, upon binding to dsRNA, PKR undergoes autophosphorylation, whereupon it phosphorylates other substrates, including eIF-2 and/or other, phosphorylatable indicator PKR protein substrates, which may be present in the mixture or added to the mixture, as indicated in the Figure. Phosphorylation of eIF-2 results in decreased protein synthesis. Hence, interference with dsRNA binding to PKR can be measured at a number of steps in the pathway -- for example, level of phosphorylation of PKR, eIF-2, or indicator protein or level of protein synthesis, as shown in the figure -- when a reaction mixture also contains the various components and reagents, such as ATP, as indicated.

D. Assay Conditions

1. Basic Assay Conditions

The claimed assay provides a method of identifying compounds that are capable of disrupting binding of a

dsRNA-binding protein or protein complex to dsRNA. Briefly, a test compound, as described above, is mixed with a dsRNA-binding protein in the presence of a fragment of dsRNA to which the dsRNA-binding protein binds. Levels of binding between the dsRNA fragment and the binding protein are measured in the presence of compound and compared to the binding level in the absence of compound. A test compound is considered to be capable of disrupting the dsRNA:dsRNA-binding protein binding, if the level of binding is substantially reduced in the presence of agent. Alternatively, the assay can be used to detect compounds that enhance binding of the dsRNA binding protein to dsRNA, simply by monitoring the reaction for increased binding levels in the presence of compound. In the context of the present invention, the terms "substantially" or "significantly" used in the context of binding levels indicate that there is a statistically significant difference between the test and reference binding levels. Statistical significance can be measured by any of a variety of methods known in the art, so long as the method is appropriate for use in the assay format.

In experiments carried out in support of the present invention, and detailed in Example 3, 30-mer RNA polynucleotides were S'-end-labeled with 32p using gamma-^{32}P ATP. ^{32}P-labeled and unlabeled oligonucleotides were annealed to produce double-stranded 30-mer RNA. In parallel experiments, the 30-mer RNA was annealed to a complementary DNA molecule to produce a double-stranded 30-mer RNA/DNA hybrid. For other experiments, 19-mer, 22-mer and 26-mer RNA duplex molecules were similarly produced.

Recombinant E3L protein (1-400 nM), produced as described in Examples 1 and 2, was added to a reaction mixture containing 0.2 nM of RNA or RNA/DNA hybrid, in the presence of the appropriate duplex RNA (or RNA/DNA hybrid) fragment. Following a reaction time of 30 min.

at room temperature, the samples were loaded onto 6% Retardation Gels; the gels were processed and subjected to autoradiography. Detection of binding was monitored by noting and quantitating the migration position of the radiolabeled RNA in the gel.

The results of experiments comparing the ability of E3L to bind double stranded 30-mer RNA oligonucleotide, compared with lack of binding to ssRNA or RNA/DNA hybrid, are discussed above. Also described is the preference of E3L for a ribonucleotide consisting of at least 30 base pairs.

2. Testing Compounds for Effects in Assay

FIGS. 5(A-D) shows a schematic of the dsRNA binding assay, where the dsRNA-binding protein is shown binding to a 30-mer, where it is appreciated that the structure illustrated as the binding protein can be a single protein or a protein complex consisting of two or more protein subunits. FIG. 5B shows a schematic of a drug or test compound binding to the center portion of a 30-mer dsRNA molecule, thereby inhibiting the ability of the dsRNA-binding protein to bind to the 30-mer. According to one hypothesis, which should not be construed to limit the invention in any way, the presence of a compound at the center of the dsRNA creates the equivalent of two 15-mers; since binding of the binding protein to smaller molecules was previously found to be considerably reduced (*c.f.*, FIG. 4), binding to this fragment, or the illustrated 10-mer fragments (FIG. 5C) is correspondingly lower. FIG. 5D illustrates the situation where the drug binds near the end of the dsRNA fragment. This construct is generally considered undesirable, in the context of the present assay, since binding to the end region may not be sufficient to measurably displace or disrupt protein binding to the polynucleotide sequence.

In experiments carried out in support of the invention, ethidium bromide, a known intercalating agent, was added to the reaction mixture containing the 30-mer dsRNA oligonucleotide, incubated for 30 min., then E3L was added and the reaction mixture was incubated for another 30 minutes at room temperature. Samples were analyzed by gel retardation experiments as described above.

FIGS. 6A and 6B show the results of these experiments.. Ethidium bromide was able to prevent E3L from binding the dsRNA at concentrations above 10 μM (FIG. 6A). Lower concentrations (3.3 μM) were effective to reduce binding when the gel also contained 3μM ethidium bromide (FIG. 6B). These results suggest that the actual K_d is well below 3μM. Results of the experiments were similar, regardless of whether ethidium bromide was added to the reaction mixture before or after incubation with E3L. In addition to providing information concerning the binding properties of ethidium bromide, this experiment also serves as an example of a paradigm that can be used to assess binding affinity to dsRNA or other polynucleotides.

In other experiments described herein, RNA binding proteins, Staufen, E3L, mPKR and hPKR, were incubated with a duplex dsRNA of 30 polynucleotides in length. Figure 12 shows that all four proteins bound to dsRNA with high affinity. hPKR bound with the highest affinity. Known RNA binding drugs were then tested for their effects on the binding of hPKR to the duplex dsRNA as shown in Fig. 14. When these drugs were tested, as expected, neomycin bound to dsRNA with the highest affinity.

Next, the assay was used to screen a biased library of novel compounds to identify compounds which bind to dsRNA and displaced hPKR. Of the 144 compounds screened, nine were able to bind to dsRNA and displaced hPKR.

The foregoing experiments illustrate the type of results obtained when a compound is found that prevents dsRNA binding protein binding to dsRNA, according to the invention. It is appreciated that, in order for the compound to be a viable drug candidate, further toxicity testing may be required. For example, toxicity of a compound selected as above might be carried out in a mammalian cell system, to ensure that the compound does not non-selectively bind other forms of polynucleotides endogenous to host cells or otherwise compromise cell metabolism in the absence of dsRNA.

3. Testing for Binding to Specific RNA Sequences

It is appreciated that all possible combinations for specific binding sites of a given length can theoretically be incorporated into a single dsRNA polynucleotide having a defined sequence. For example, for a three-nucleotide binding site, there are 4^3=64 possible combinations which can be incorporated into a 34-mer dsRNA sequence (FIG. 7A). An example of such a sequence is presented in FIG. 7A, where all possible 3-mers are represented either on "+" or the "-" strand. For purposes of the screening assay, the 34-mer sequence can be divided between several oligonucleotides. For example, it could be nearly equally divided between 2 oligonucleotides, each of which has a unique set of the three-nucleotide binding sites (FIG. 7B). Flanking sequences are provided to secure sufficient size of the dsRNA for binding. Fig. 7F and 7G show two oligonucleotides with flanking sequences which together provide all 64 possible combinations for a three-nucleotide binding site. The oligonucleotide of 7F was biotin labeled and used in scintillation proximity assays. This 32-mer circle contains all 64 possible combinations of 3-mer sequences without redundancy. The duplex oligonucleotide in Fig. 7A can be viewed as a 32-mer circle to simplify analysis. When the duplex is

viewed as a 32-mer circle, any two or more oligonucleotides can be identified which together would provide all 64 possible combinations of three nucleotides.

Similarly, all the possible 4^4=256 4-nucleotide binding sites could be accommodated among 8 oligonucleotides (Fig. 11). In another embodiment, two or more binding sites per oligonucleotide can be introduced, as represented on FIG. 7C. In this case the 34-mer sequence can be divided between more than 2 oligonucleotides. For example the 34-mer sequence can be divided between 4 nucleotides, each carrying 2 copies of approximately 25% of the 34-mer sequence (FIG. 7C). The nucleotide sequence in one of the copies in each oligonucleotide can be rearranged in order to facilitate precise annealing of the strands of the oligonucleotides. In order to perform the binding sequence specificity studies of the compounds, the 34-mer sequence could be divided further between more oligonucleotides, each carrying single (FIG. 7D) or multiple (FIG. 7E) pieces of the 34-nucleotide sequence.

Initial screening of the compounds (prescreen) can be performed with oligonucleotides which incorporate many binding sites, such as the polynucleotides represented in FIGS. 7B and C. Subsequent characterization of the compound sequence specificity is performed with oligonucleotides similar to represented on Fig. 7D and E, which are designed as subsets of binding sites of nucleotides which bound certain compounds at the prescreen step. Simultaneous RT-PCR studies can be carried out in order to establish the identity of the binding sites of the dsRNA sequence-specific compounds.

III. dsRNA Binding Agents

A. Small Molecule Concatemeric dsRNA Binding Agents

According to an important feature of the present invention, the screening assay described above can be used to identify (i) compounds that bind with relatively high affinity (*e.g.*, K_d micromolar or above) to dsRNA and thereby inhibit binding of binding proteins to the RNA, (ii) compounds that bind with sequence specificity to duplex dsRNA, or (iii) compounds that bind specifically to dsRNA structures without sequence specificity. Both sequence specific and non-specific compounds can be used to form concatemeric agents with enhanced specificity for dsRNA, in accordance with the present invention. That is, agents can be formed from solely sequence non-specific compounds, from solely sequence-specific compounds, or from a combination thereof. Both types of compounds can be identified using the screening assays described herein and, based on their characteristics, can be catalogued so that custom concatameric duplex dsRNA binding agents can be formed.

More particularly, according to this aspect, the invention can be seen to describe and encompass dsRNA binding agents which bind with base sequence specificity to dsRNA, as well as those that bind with base sequence non-specificity. Such agents are designed using the information derived from the binding assay, as follows. When a target dsRNA molecule is identified (as by selecting a particular virus whose genome is known to be regulated by a dsRNA binding protein), the sequence of the RNA genome, or portion thereof, may be identified. Using the libraries of compounds defined by screening in the assays described in the previous sections, a concatemer of two or more small molecule dsRNA binding compounds is designed. One or more of the compounds may be selected for its ability to bind with sequence

selectivity to a particular 3-16, or preferably a 3-8, base pair sequence which is also found in the target genome. A second compound is selected to bind to an adjacent or nearby region (within about 20 bases) in the viral genome, and that second compound is linked to the first compound using appropriate linking chemistry, including but not limited to linking using a polynucleotide linker, a peptide linker, a polysaccharide spacer, or the like, as discussed below.

Alternatively, according to an important aspect of the present invention, the concatemer can be formed of compounds that bind sequence non-specifically to dsRNA. Such combinations are particularly useful in combating infectious agents of unknown etiologies and/or identities. More preferably, the agent will consist of a mixture of sequence-specific and sequence-non-specific molecules.

The resulting agent is preferably a linear, non-oligonucleotide concatemer formed by covalently linking first and second compounds. As mentioned above, at least one compound in the concatemer may be selected to provide base-specific binding to a 3-8 base pair region in the target dsRNA. Other compounds selected for forming the concatemer may exhibit sequence non-specific binding or sequence specific (base-specific) binding.

As mentioned above, an advantage to using at least one concatemeric subunit which exhibits sequence non-specific binding is that this potentially broadens the range of organisms which can be targeted by the agent. Such broad spectrum agents are particularly important in combating viral pathogens having unknown and/or highly-mutated genomic sequences (such as influenza virus).

An advantage to this concatemeric approach to producing anti-viral agents rests, in part in the following analysis. That is, any particular RNA-binding small molecule screened in the assay may only recognize a 2-4 base pair site. Even if the recognition is quite

specific, the molecule may be toxic to the host cell, because, while mammalian cells do not generally have long stretches of duplex RNA, short stretches do exist, for example in the hairpin regions of transfer RNA (tRNA). However, potential toxicity of RNA-binding drugs may be greatly reduced by creating dimers, trimers, or multimers with these compounds, since such concatemeric agents will bind to longer, presumably viral, regions.

Further, from theoretical considerations of the free energy changes accompanying the binding of drugs to dsRNA, the intrinsic binding constant of a dimer should be the square of the binding constant of the monomer. Trimerization, which theoretically should yield binding affinities that are the cube of the affinity of the homomonomeric subunit or the product of affinities of the heteromonomeric subunits, has yielded compounds with affinities as high as 10^{-12}M in DNA systems (Laugaa, *et al.*, Biochemistry 23:1336, 1985).

As a hypothetical example, if a relatively weak dsRNA-binding drug, drug X, which binds a 4 bp site with an affinity of 2×10^{-5} M was dimerized, the bis-X drug would now recognize an 8 bp site with a theoretical affinity of 4×10^{-10} M. The difference in affinity between the monomer X and the bis-X form is 200,000-fold.

There are two immediate ramifications of dimerization (or multimerization) of monomeric drugs with moderate toxicity. First, the concentration of drug needed is lowered because of the higher affinity, so that even relatively toxic molecules can be used as drugs. Second, since toxicity is likely linked to the average number of drug molecules bound to the genome and/or to transcript or ribosomal RNA's, as specificity for longer dsRNA domains is increased by increasing the length of the binding site, toxicity is decreased. Nonetheless, it is preferred to test compounds in a cell

toxicity assay, prior to selection as potential therapeutics.

In the present context, it is appropriate to point out that the screening method of the present invention creates a "bank" of compounds with high combinatorial potential as dsRNA binding agents. If 50 to 100 dsRNA-binding monomeric ligands are discovered, this represents a potential to bind to 250-500 high affinity sites and 1000-2500 moderately high affinity sites. Thus, the probability of finding a number of high affinity drug binding sites that match medically significant target sites is good. Furthermore, heterodimeric drugs can be designed to match RNA target sites of 8 or more bp, lending specificity to the potential pharmaceuticals.

As discussed above, once the sequence preferences of a number of molecules are known, the information may be used to design oligomeric molecules, or concatemers, (homopolymers or heteropolymers) with substantially greater sequence specificity and substantially higher binding affinity. For example, if a dsRNA-binding molecule, X, binds a 4 bp sequence 5'-ACGU-3'/5'-ACGU-3' with an equilibrium affinity constant of 2×10^{-5} M, then the dimer of X, X_2, should bind the dimer of the sequence, 5'-ACGUACGU-3'/5'-ACGUACGU-3' (SEQ ID NO: 2), with an equilibrium affinity constant of $(2 \times 10^{-5}\ M)^2 = 4 \times 10^{-10}$ M. The dsRNA-binding dimer molecule, X_2, recognizes an 8 bp sequence, conferring higher sequence specificity, with a binding affinity that is theoretically 200,000-fold higher than the RNA-binding monomer, X.

The same argument can be extended to trimer molecules: the trimer of X, X_3, would bind a 12 bp sequence, 5'-ACGUACGUACGU-3'/5'-ACGUACGUACGU-3'(SEQ ID NO: 3), with a theoretical equilibrium affinity constant of 8×10^{-15}M. RNA-binding polymers constructed using the above-mentioned approach may be homo- or heteropolymers of the parent compounds or oligomeric compounds

composed of mixed subunits of the parent compounds. Homopolymers are molecules constructed using two or more subunits of the same monomeric RNA-binding molecule. Heteropolymers are molecules constructed using two or more subunits of different monomeric RNA-binding molecules. Oligomeric compounds are constructed of mixed pieces of parent compounds and may be hetero- or homomeric.

RNA-binding subunits can be chemically coupled to form heteropolymers or homopolymers. The subunits can be joined directly to each other, as in the family of distamycin molecules, or the subunits can be joined with a spacer molecule, such as carbon chains or peptide bonds. The coupling of subunits is dependent on the chemical nature of the subunits: appropriate coupling reactions can be determined for any two subunit molecules from the chemical literature. The choice of subunits will be directed by the sequence to be targeted and the data accumulated through the methods discussed in Section VI.B of this application.

B. dsRNA Modifying Reagents

According to an important feature of the present invention, it is appreciated that for some applications it may be desirable to equip concatemeric agents formed as discussed above with additional chemical reactivity that will selectively (over all other molecules in a cell or tissue) chemically modify the dsRNA, when the binding agent binds to the dsRNA or comes in very close proximity to the dsRNA. Here, the dimer/multimer portion of the concatemeric molecule directs the binding to the dsRNA as described above. The reactive portion of the molecule does not necessarily contribute to binding to the RNA. The reactive moiety is preferably linked to the linear multimeric concatemer using a linker strategy akin to that for making the dimers/multimers as described above. Irreversible

chemical modification of the dsRNA in accordance with this embodiment of the invention is designed effectively to interfere with some important or essential function of the RNA and thus render it less able to participate in its normal functions, for example viral replication.

There are a number of reactive chemistries that can be used to construct such molecules. Some preferred embodiments not exclusive of others include the following: (1) covalent modification (*i.e.*, alkylation of guanine bases by nitrogen mustard reagents or pro-reactive chylopropyl functionalized polyheteroaromatic reagents), (2) interstrand cross-linking (*i.e.*, by psoralens), (3) transesterification cleavage (*i.e.*, by Lewis acid metal chelate complexes), and (4) cleavage by other activated species (*i.e.*, ene-diynes; activated higher valency metallo-oxo complexes). An advantage of molecules formed according to this aspect of the invention is to provide an enhanced function or response over that which can be achieved by binding of the dimer/multimer alone.

IV. Utility

The RNA:protein assay of the present invention has been designed to screen for compounds that bind a full range of RNA sequences that vary in length as well as complexity. RNA-binding molecules discovered by the assay have potential usefulness as either molecular reagents, therapeutics, or therapeutic precursors. Sequence-specific or sequence non-specific dsRNA-binding molecules are potentially powerful therapeutics for essentially any disease or condition that involves dsRNA. Examples of test sequences for the assay include: a) binding sequences of factors involved in the maintenance or propagation of infectious agents, especially viruses, bacteria, yeast and other fungi, b) sequences causing the inappropriate expression of

certain viral genes, and c) sequences involved in the replication of rapidly growing cells.

Molecules identified by the present assay may be particularly valuable as lead compounds for the development of congeners having either different specificity or different affinity.

One advantage of the present invention is that the assay is capable of screening for binding activity directed against dsRNA generally (*e.g.*, dsRNA structural specific or dsRNA sequence non-specific compounds), or specific dsRNA sequences (dsRNA sequence specific compounds). One category of target sequences includes those present in medically or agriculturally significant pathogens (viruses and virioids, respectively).

The assay can also be used to screen for molecules demonstrating sequence preferential binding to determine the sequences with highest binding affinity and/or to determine the relative affinities between a large number of different sequences. There is usefulness in taking either approach for detecting and/or designing new therapeutic agents.

Also forming part of the present invention are methods of treating or preventing viral or virioid infections. Such methods generally include administering to an infected subject or to a subject in danger of infection dsRNA binding compounds or concatemers formed from compounds selected in accordance with the screening methods described herein. Although a number of viruses can be treated using these methods, including unknown or mutant viruses of unknown genomic sequences, as discussed above, viruses or virioids having known ssRNA or dsRNA genomes also can be treated as discussed in the Section that follows.

A. Agents: Target Organisms

Compounds discovered using the assay methods described in conjunction with the present invention as

well as concatemer compounds constructed in accordance with the invention are particularly suited for use in combating diseases having viral etiologies, for the reasons described above. Few effective viral therapeutics are currently available. Furthermore, with the accumulation of sequence data on all biological systems, including viral genomes, cellular genomes, pathogen genomes (bacteria, fungi, eukaryotic parasites, etc.), the number of target sites for dsRNA-binding drugs will increase greatly in the future.

There are numerous methods for identifying medically significant target sequences for dsRNA-binding drugs, including, but not limited to, the following. First, medically significant target sequences are found in pathogens of the biological kingdoms, particularly in viral pathogens. Second, a target region of the pathogen is identified, such as in the ssRNA or dsRNA sequences of the virus. Specific target sequences are identified that affect the expression or activity of the genomic RNA molecules, such as sites involved in viral replication.

As mentioned above, RNA viruses may be particularly susceptible to anti-viral methods and compositions described herein. Table I lists a number of potential target pathogens, including human, animal and plant pathogens.

Table 1
Pathogenic RNA Viruses

Genome	Family	Genus	Species
Single Stranded RNA	*Picornaviridea*	Enterovirus	Poliovirus 1,2,3
Positive Stranded			Coxsackieviruses A1-22, A24
Non-Segmented			Human echoviruses 1-7, 11-27, 29-33
7.2-8.4 Kb			Human enteroviruses 68-71
			Swine vesicular disease virus
			Porcine enteroviruses 1-8
			Bovine enteroviruses 1-7
		Cardiovirus	Encephalomyocarditis Virus

Genome	Family	Genus	Species
		Rhinovirus	Human rhinovirus 1-100, 1A, 1B, Hanks
			Bovine rhinoviruses 1-3
			Equine rhinoviruses 1 and 2
		Aphthovirus	Foot and mouth disease viruses O, A, C, SAT 1-3
		Hepatovirus	Hepatitis A
Single Stranded RNA	*Calciviridae*	Calicivirus	Norwalk (Southhampton, Snow Mountain, Hawaii, Taunton)
Positive Stranded			Hepatitis E Virus (unclassified)
Non-Segmented			Vesicular exanthema virus of swine
7.4-7.7 Kb			Canine calicivirus
			Bovine enteric calcivirus
			Porcine enteric calcivirus
			Mink calcivirus
			Fowl calcivirus
Single Stranded RNA	*Astroviridae*	Astrovirus	Human Astroviruses 1-5
Positive Stranded			Bovine Astroviruses 1 and 2
Non-Segmented			Porcine Astrovirus
7.2-7.9 Kb			Canine Astrovirus
Single Stranded RNA	*Togaviridae*	Alphavirus	Eastern equine encephalitis virus
Positive Stranded			Venezuelan equine encephalitis virus
Non-Segmented			Sindbis virus (Ochelobo and Babanki)
9.7-11.8 Kb			Chikungunya virus
			O'nyong-nyong
			Igbo Ora virus
			Ross River virus
			Mayaro virus
			Barmah Forest virus
			Eastern equine encephalitis virus
			Venezuelan equine encephalitis virus
			Eastern equine encephalitis virus
			Western equine encephalitis virus
			Getah
		Rubivirus	Rubella virus
Single Stranded RNA	*Flaviviridae*	Flavivirus	Yellow fever virus
Positive Stranded			Dengue viruses 1-4
Non-Segmented			Japanese encephalitis virus
9.5-12.5 Kb			West Nile virus
			Murray Valley encephalitis
			Rocio virus
			Tick-borne encephalitis viruses
			European tick-borne encephalitis viruses
			Far Eastern tick-borne encephalitis viruses
			Russian Spring-Summer encephalitis viruses
			Kyasanur forest disease virus

Genome	Family	Genus	Species
			Omsk hemorrhagic fever virus
			Luoping ill virus
			Powassan virus
		Pestivirus	Bovine viral diarrhea virus
			Hog cholera virus
			Border disease virus of sheep
		unnamed	Hepatitis C Virus
			Hepatitis G Virus
Single Stranded RNA	*Coronaviridae*	Coronavirus	Human coronaviruses 229-E, OC43, others
Positive Stranded			Infectious bronchitis virus of fowl
Non-Segmented			Turkey blue comb disease
20-30 Kb			Transmissible gastroenteritis virus of swine
			Hemagglutinating encephalomyelitis virus of swine
			Porcine epidemic diarrhea virus
			Calf coronavirus
			Feline infectious peritonitis virus
			Feline enteric coronavirus
			Canine coronavirus
		Torovirus	Enteric and respiratory virus
			Berne virus (horses)
			Breda virus (calves)
			Bovine respiratory torovirus
			Feline torovirus
Single Stranded RNA		Arterivirus	Lelystad virus (porcine reproductive and respiratory syndrome virus)
Positive Stranded			
Non-Segmented			VR2332 (swine)
15 Kb			Equine arteritis virus
Single Stranded RNA	*Paramyxoviridae*	Paramyxovirus	Human parainfluenza virus 1 and 3
Negative Stranded			Bovine parainfluenza virus 3
Non-Segmented			Sendai virus
16-20 Kb		Rubulavirus	Human mumps
			Human parainfluenza viruses 2, 4a and 4b
			Newcastle disease virus of chickens
			Avian paramyxovirus 2 (Yucaipa virus)
			Avian paramyxovirus 3,4,5 (Kunitachi virus) 6,7,8,9
			Porcine rubulavirus (la-Piedad-Michoacan-Mexico)
			Simian parainfluenza virus
		Morbillivirus	Measles virus
			Canine distempter
			Rinderpest virus
			Peste-des-petitis-ruminants virus (goats and

Genome	Family	Genus	Species
			sheep)
			Equine morbillivirus
		Pneumovirus	Human respiratory syncytial virus
			Bovine respiratory syncytial virus
			Pneumonia virus of mice
Single Stranded RNA	*Rhabdoviridae*	Vesiculovirus	Vesicular stomatitis virus (VSV-1,2,3 and 4)
Negative Stranded			Chandipura virus
Non-Segmented			Piry virus
13-16 Kb			Isfahan
		Lyssavirus	Rabies virus
			European bat viruses 1 and 2
			Mokola virus
			Duvenhage virus
			Lagos bat virus
		Ephemerovirus	Bovine ephemeral fever virus
SS, Negative, 8.9 Kb	*unclassified*	Bornavirus	Borna Disease Virus
Single Stranded RNA	*Filoviridae*	Filovirus	Marlburg virus
Negative Stranded			Ebola virus
Non-Segmented			Ebola virus Zaire
19.1 Kb			Ebola virus Sudan
Single Stranded RNA	*Orthomyxoviridae*	Influenza A,B	Influenzavirus A,B (Humans and many animals)
Negative and some ambisense		Influenza C	Influenzavirus C (humans and swine)
Segmented			
10-13.6 Kb			
Single Stranded RNA	*Bunyaviridae*	Bunyavirus	Bunyamwera virus
Negative and some ambisense			Bwamba virus
Segmented			Oriboca virus
11-21 Kb			Oropouche virus
			Guama virus
			LaCrosse virus
			Jamestown Canyon virus
			California encephalitis virus
			Showshoe hare virus (rabbits to humans)
			Tahyna virus
			Akabane and Aino (animal)
		Phlebovirus	Sandfly fever-Naples virus
			Sandfly fever-Sicilian virus
			Rift Valley fever virus
		Nairovirus	Crimean-Congo hemorrhagic fever virus
		Hantavirus	Hantaan virus
			Seoul virus (hemorrhagic fever with renal syndrome)
			Sin Nombre virus (acute respiratory distress

Genome	Family	Genus	Species
			syndrome)
			Puumala virus (nephropathia epidemica)
Single Stranded RNA	*Arenaviridae*	Arenavirus	Lymphocytic choriomeningitis virus
Negative and some ambisense			Lassa virus
Segmented			Machupo virus (Bolivian hemorrhagic fever)
10-14 Kb			Junin virus (Argentine hemorrhagic fever)
			Guanarito virus (Venezuelan hemorrhagic fever)
Double Stranded RNA	*Reoviridae*	Reovirus	Human reovirus 1,2,3 is not very pathogenic
Positive sense			Avian reoviruses
Segmented		Orbivirus	Orungo virus (febrile illness in Nigeria and Uganda)
16-27 Kb			Kemerovo virus (febrile illness in Russia and Eygpt)
			Blue tongue viruses 1-25
			African horsesickness viruses 1-9
			Equine encephalosis virus
		Rotavirus	Human rotavirus, Groups A and B
			Porcine Group B and E rotaviruses
			Fowl group D rotaviruses
			Group F avian rotaviruses
		Coltivirus	Colorado tick fever virus
			Eyach virus (humans)
Double Stranded RNA	*Birnaviridae*	Birnavirus	Infectious bursal disease virus (domestic fowl)
Positive Sense			Infectious pancreatic necrosis virus of fish
Segmented			
5.7-5.9 Kb			
Single Stranded RNA		Deltavirus	Human Hepatitis Delta Virus (defective)
Circular			
Negative Sense			
1.7 Kb			
Single Stranded RNA		Virioids	Potato spindle tuber virioid
Circular			Avocado sunblotch virioid
250-350 bp			Grapevine virioid
infectious raw RNA			Citrus exocortis virioid
			Grapevine yellow speckle virioid
			Hop shunt virioid
			Dapple apple virioid
			Tomato apical shunt virioid
			Other plant virioids

B. Method of treating viral infections

As mentioned above, compounds discovered in accordance with the screening methods of the present invention are particularly useful in preventing and/or treating viral infections caused by RNA viruses (viruses having ssRNA or dsRNA as a genome).

According to this aspect of the invention, the presumed viral pathogen may or may not be examined to determine the sequence of its genome, and compounds are sought and selected that are either specific (if determined) or nonspecific (if not determined) for binding to a particular base pair sequence found within the genome.

An important aspect of the invention is a broad-spectrum anti-viral agent. Because long stretches of dsRNA occur only in virus-infected cells, a compound which binds with base pair sequence non-specificity to dsRNA is useful as a broad-spectrum antiviral.

A concatemer is formed from test dsRNA binding compounds, in any combination of sequence-specific and/or sequence non-specific molecules, as required. The compounds will bind to adjacent 3-16 or 3-8 base pair regions. In this context, the term "adjacent" means within about 20 base pairs of each other.

The concatemer is then formed from the two binding molecule subunits. Following the appropriate cell and animal toxicity tests, the compound is formulated into an appropriate pharmaceutical excipient and subjected to regulatory review. Appropriate excipients, dosing and dosing schedules and modes will vary, according to the type and size of compounds forming the concatemer; such determinations will be well within the skill of persons skilled in the art, once the composition of the concatemer is known. The subject is then treated with a therapeutically effective amount of the concatemer (or single binding agent).

All references and patents specifically cited in this application are herein incorporated by reference in their entireties and are specifically incorporated by reference with respect to the context in which they are cited.

The following examples illustrate, but in no way are intended to limit the present invention.

EXAMPLES

Example 1

Cloning and Expression of dsRNA Binding Domains of E3L, Staufen, hPKR, mPKR.

A number of dsRNA binding proteins are described in the literature (Burd C.G., *et al.*, Science 265: 615-621, 1994). Since different proteins can considerably differ in their activity, solubility, yield, stability, and other factors influencing performance in the assays, several RNA-binding proteins were expressed.

Vaccinia virus E3L, Drosophila melanogaster staufen, and human and mouse PKR proteins have similar domains recognizing dsRNA. An article disclosing the RNA binding domains of these proteins was provided by St. Johnston, *et al.* (St Johnston D., *et al.*, Proc. Natl. Acad. Sci. USA 89: 10979-10983, 1992). dsRNA binding domains (dsRBD) of these proteins were expressed as glutathione S-transferase (GST) fusions in E.coli (Fig. 1) (Chang H.-W. and Jacobs B. L. , Virology 194: 537-547, 1993; Burd C. and Dreyfuss G., Science 265: 615-621, 1995). DNA fragments encoding dsRBDs of these proteins were obtained by PCR. E3L dsRBD was obtained by using vaccinia virus DNA as a template as described in Example 2. Staufen dsRBD was obtained using as a template dsRBD3 plasmid DNA containing the staufen dsRBD (St. Johnston D., *et al.*, Proc. Natl. Acad. Sci. USA 89: 10979-10983, 1992; St. Johnson D., et al., Cell 66: 51-63, 1991). Human and mouse PKR dsRBDs were obtained

using human and mouse cDNA and the Advantage cDNA PCR kit. PCR was performed according to the manufacturer's instructions.

The following DNA oligonucleotides were used as primers in the PCR reactions. Restriction endonuclease sites are underlined:

E3L:

Forward:

GCGGGATCCTTTGATGATGTTATTCCGG (*BamHI*) (SEQ ID NO:4)

Reverse:

GCGGAATTCAGAATCTAATGATGACG (*EcoRI*) (SEQ ID NO:5)

Staufen:

Forward:

GCGCAGATCTATGGATGAGGGTGACAAGAA (*BglII*) (SEQ ID NO:17)

Reverse:

GCGGAATTCACTTGGTGGGCGTAAGG (*EcoRI*) (SEQ ID NO:18)

hPKR:

Forward:

GCGCAGATCTGCTGGTGATCTTTCAGC (*BglII*) (SEQ ID NO:11)

Reverse:

GCGCGATATCTAACCATTCATAAGCAACGAA (*EcoRV*) (SEQ ID NO:12)

mPKR:

Forward:

GCGCAGATCTGCCAGTGATACCCCAG (*BglII*) (SEQ ID NO:13)

Reverse:

GCGCGATATCTAATTACTTGTCATAGACGAG (*EcoRV*) (SEQ ID NO:14)

pGEX-2T E.coli plasmid expression vector was used to clone the PCR fragments. The vector contains the

glutathione binding moiety of the Schistosoma japonicum glutathione S-transferase (GST) under the control of the strong chimeric tac promoter (Smith D. B., *et al.*, Gene 67: 31-40, 1988). PCR fragments are cloned in lieu of the GST moiety in-frame with the GST open reading frame, and dsRBDs were expressed as GST carboxy-terminal fusions. To this end, hPKR and mPKR PCR fragments were cleaved with BgLII and EcoRV restriction endonucleases, and cloned into the BamHI/SmaI cut vector. Staufen PCR fragment was cut with Bg1II and EcoRI restriction nucleases and cloned into BAM HI/EcoRI digested pGEX-2T vector. The cloning was performed using T4 DNA ligase, as follows.

Plasmid and insert DNA are mixed at a ratio of 1:10 and ligated with T4 DNA ligase overnight at 10 degrees C in 50 mM Tris-HCl pH 7.5, 7 mM MgCl2, 1 mM DTT, 1 mM ATP, 1 ug DNA and 50 U/ml enzyme. The ligation mixture was used to transform E. coli (Epicurian Coli XL1-Blue super competent cells, Statagene, La Jolla, CA), according to the manufacturer's instructions. Positive colonies were isolated on the plates with 100 ug/ml ampicillin and plasmids purified using WizardTM minipreps DNA purification systems (Promega, Madison, WI). Purified plasmids were checked for correct inserts using restriction endonuclease analysis.

Cultivation of E.Coli strains expressing dsRBD of E3L, staufen, hPKR and mPKR proteins was done essentially as described for the strains harboring pGEX-2T vector or its derivatives (Smith and Johnson, Gene 67:31, 1988). Overnight cultures of the E.Coli strains were diluted 1:10 in 600ml of LB medium containing 100mkg/ml ampicillin and cultured at 30-33°C in a controlled environment incubator shaker (New Brunswick Scientific, Edison, NJ) with 150 rpm agitation. Cells were grown for 1 hour before adding IPTG (isopropyl-β-thiogalactosidase; GibcoBRL, Gaithersburg, MD) to 0.2 mM

concentration, and harvested after further 5 hour growth. Samples of cells were taken before and after induction. Cells were centrifuged in an Eppendorf microfuge at 5000 rpm for 2 minutes, resuspended in the initial volume of PBS and centrifuged again at the same conditions. Cell pellets were resuspended in 1x sample buffer (0.25M Tris-HCl, pH 6.8, 20% Glycerol, 0.1% Bromophenol blue, 2.5% β-mercaptoethanol, 5% SDS), and incubated at 100°C for 5 minutes. Cell proteins were separated by SDS-PAGE in 6-20% Tris-Glycine gels (Novex, San Diego, CA) according to the manufacturer's instructions. A band, corresponding to protein of 37 KD, which is close to the expected size for both GST-staufen and GST-E3L fusion proteins (35 KD), was detected in the induced cells, but not in the uninduced cells.

Similarly, bands corresponding to proteins of 47 KD and 50 KD were detected in total cell lysates of induced E.Coli GST-mPKR and GST-hPKR, but not in the lysates of uninduced cells.

GST-E3L, GST-staufen, GST-mPKR and GST-hPKR fusion proteins harboring the respective double stranded binding domains are further referred to as E3L, staufen, mPKR and hPKR.

Example 2

Purification of E3L, staufen, mPKR and hPKR

Cell cultures generated as described in Example 1 were pelleted and resuspended in 1:100 culture volume of Dulbecco PBS (GibcoBRL, Gaithersburg, MD). Cells were disrupted on ice by 4x30 seconds rounds of sonication at the highest output of the low switch of the Braun-Sonic 2000 sonicator. Triton X-100 (Sigma, St. Louis, MO) was added to 1% concentration and the lysates were centrifuged at 5°C for 30 minutes at 19,000 rpm in the JA-20 rotor. Some E3L and staufen protein was found in the supernatant, which was designated as the "PBS

fraction." The majority of the E3L and staufen protein remained in the pellet. Both mPKR and hPKR were predominantly soluble, and practically all hPKR and most of the mPKR were found in the supernatant.

E3L present in the pellet was recovered by extraction with 0.5M NaCl - 40mM Tris-HCl pH 7.5, for 1.5-2 h at room temperature. Soluble and insoluble fractions were separated by centrifugation at room temperature for 30 minutes at 19 000 rpm in the JA-20 rotor. E3L recovered from the supernatant was designated the "pH 7.5 fraction". The pellet was further extracted with 0.5M NaCl - Tris-HCl pH 8.0, followed by centrifugation. The supernatant was designated the "pH 8.0 fraction". Further extraction of E3L protein from the pellet could be achieved at more basic pH, for example with 40mM carbonate buffer pH 9.5.

E3L pH 7.5 and pH 8.0 fractions were diluted 1:2 with water, and the diluted fractions as well as the PBS fraction were incubated for 1 h at 4C with 1/20 volume of Glutathione Sepharose 4B beads (Pharmacia Biotech, Sweden). Beads were washed 3 times with 20x volumes of the each fraction buffer, and E3L protein was eluted from the beads with 5 mM reduced glutathione - 50 mM Tris-HCl, pH 8.0. The E3L protein obtained in the eluates was essentially pure. Fractions containing E3L protein were pooled and were dialyzed against 100 volumes of 0,5M NaCl - 40 mM tris-HCl, pH 8.0, 2mM Ditiothreitol, 0.1% Triton-X-100, 20% Glycerol. No difference in E3L dsRNA binding activity was detected between the fractions (data not shown). The obtained protein preparations were aliquoted and frozen at -80°C.

Staufen, mPKR and hPKR supernatant fractions were incubated for 1 hour at 4°C with 1/20 volume of Glutathione Sepharose 4B beads (Pharmacia Biotech, Sweden). The beads were washed 3 times with 20x volumes of PBS buffer, and proteins were eluted from the beads with 5 mM reduced glutathione - 50 mM Tris-HCl, pH 8.0.

The hPKR and mPKR proteins obtained in the eluates were essentially pure, and staufen was approximately 50% pure, as shown by polyacrylamide gel electrophoresis. Fractions containing staufen were pooled and dialyzed against 100 volumes of 0,5M NaCl, 40 mM tris-HCl, pH 8.0, 2mM Dithiothreitol, 0.1% Triton-X-100, 25% Glycerol. Fractions containing mPKR or hPKR were dialyzed against 100 volumes of 0.1 M NaCl, 40 mM tris-HCl, pH 8, 2mM Ditiothreitol, 0.1% Triton X-100, 20% Glycerol. The obtained protein preparations were aliquoted and frozen at -80°C.

Example 3

RNA Binding Assay

E3L binds double stranded RNA, but does not bind ssRNA or RNA/DNA hybrid (Kiong and Shuman, *Virology* 217:227, 1996; See also FIG. 3). RNA oligonucleotides for the E3L-RNA binding experiments were synthesized at Cruachem Inc. Dulles, VA.

1T 5'-GCUGGGUUCUUGCGGUGGCUCGUGCUUUCG-3' (SEQ ID NO: 6)
1B 3'-CGACCCAAGAACGCCACCGAGCACGAAAGC-5' (SEQ ID NO:16)
19T 5'-UUCUUGCGGUGGCUCGAGC (SEQ ID NO: 7)
22T 5'-GGUUCUUGCGGUGGCUCGUGCU (SEQ ID NO: 8)
26T 5'-UGGGUUCUUGCGGUGGCUCGUGCUUU (SEQ ID NO: 9)

The 30-mer DNA oligonucleotide 1TD, with the same sequence as the RNA oligonucleotide 1T, was synthesized at Keystone Laboratories, Inc., Redwood City, CA. The 1TD 5'-GCTGGGTTCTTGCGGTGGCTCGTGCTTTCG-3' (SEQ ID NO: 10) Oligonucleotide 1B was end-labeled with gamma-^{32}P ATP (Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) according to the manufacturers instructions. ^{32}P labeled 1B and unlabeled oligonucleotides 1T,19T, 22T or 26T were annealed in a reaction mixtures containing 700nM of ^{32}P labeled 1B and 800nM of an unlabeled 1T, 1TD, 19T, 22T or 26T, 15mM Tris-HCl, pH 7.5, 400mM NaCl,

2.5mM EDTA. The sample was slowly cooled from 70C to room temperature during 2 h. Annealing of 1T and 1B oligonucleotides produced perfectly double-stranded 30-mer RNA. Annealing of 1TD and 1B oligonucleotides produced perfectly double-stranded 30-mer RNA/DNA hybrid. Annealing of 19T, 22T and 26T with 1B resulted in RNA, which had 19, 22 and 26 base pairs of perfectly double-stranded RNA structure, and are further referred to as 19- 22- and 26- mer.

Binding of E3L to oligonucleotides was performed in a reaction mixture containing 0.2 nM of RNA or RNA/DNA hybrid, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM NaCl, 0.1% Triton X-100, 5% Glycerol, 2 mM Ditiothreitol and 1-400 nM of E3L protein. The reaction was allowed to proceed for 30 min. at room temperature, after which 70% glycerol was added to 15% concentration and the samples were loaded onto 6% Retardation Gels (Novex, San Diego, CA). The gels were run in Novex XCell™ Mini-Cell filled with 0.5x TBE buffer at 100v for 30-45 min and dried using SGD-40 gel dryer (Savant Instruments, Inc., Farmingdale, N. Y.). Gels were exposed overnight with the Scientific Imaging film (Eastman Kodak Co., N. Y.).

E3L was able to bind double stranded 30-mer RNA dsRNA fragment polynucleotide (SEQ ID NO: 6) with a K_d of approximately 4-12 nM (FIG. 2), whereas it did not effectively bind ssRNA (^{32}P labeled 1B) or RNA/DNA hybrid even at the concentrations as high as 400 nM (Fig. 3). Apart from E3L binding of RNA being strictly double stranded structure specific, it also requires a minimum size of the dsRNA structure for efficient binding. E3L binding of a 30-mer was approximately 50-100 times more efficient than a 19-mer, and binding of the 22-mer and 26-mer was only slightly better than a 19-mer (Fig. 4).

Example 4

Testing Compounds for Effects on dsRNA:dsRNA Binding Protein Binding

Ethidium bromide was added to a reaction mixture containing a 30-mer dsRNA oligonucleotide, as described in Example 2, incubated for 30 min, then E3L was added and the reaction mixture was incubated for another 30 minutes at room temperature. Samples were analyzed by gel retardation experiments as described above. Ethidium bromide was able to prevent E3L from binding the dsRNA at concentrations above 10 μM (FIG. 6A). Even a lower concentration of 3.3 μM was efficient if the gel contained Ethidium bromide (FIG. 6B), indicating that the actual K_d is well below 3mM. Results of the experiments were similar, regardless of whether ethidium bromide was added to the reaction mixture before or after incubation with E3L (data not shown).

Example 5

Preparation of dsRNA for Initial Gel-Based and HTS Assays

Both synthetic oligonucleotides and dsRNA prepared using molecular biology techniques can be used in the assays described herein. Synthetic complimentary ribooligonucleotides, preferably 15-30 bases long, can be annealed to produce dsRNA for the experiments. Depending on the type of the assay, oligonucleotides can be unmodified or modified at specific positions with biotin, fluorophor or quencher groups. Synthetic oligonucleotides are generally used at the pre-screen stage to detect dsRNA-binding molecules and to differentiate between sequence-specific and sequence non-specific RNA binding molecules. RNA molecules are also synthesized in *in vitro* transcription systems in which a hairpin oligoribonucleotide is synthesized from a DNA template (i.e., the linear sequence includes the top strand of the stem, the loop sequences, then the

bottom complementary strand of the stem). Using this type of strategy allows for the synthesis of self-annealing dsRNA oligonucleotides with specific internal 4 bp test sites. For oligonucleotides with mixed base test sites, a partial hairpin sequence is transcribed from a DNA-template (i.e., the top strand stem, the loop, and part of the bottom strand stem), allowed to anneal and used to prime synthesis of the remaining stem structure. This strategy allows for the synthesis of mixed fully complementary test sequences and also facilitates radiolabeling, if needed for the assay protocol used. Specific methods for effecting the above-described synthetic paradigms are well known in the art (e.g., Ausubel, F.M., *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, Media, PA).

Example 6

Scintillation Proximity Assay

Scintillation proximity assay (SPA) is a well-known assay (see, e.g., U.S. Patent No. 4,382,074 and 4,271,139, herein incorporated by reference). In this assay, as shown in Fig. 9, the spatial proximity of a radiolabeled atom determines the scintillation of a coated bead. When a radioactive atom decays it releases sub-atomic particles, such as electrons. The distance these particles will travel through water is limited and is dependent on the nature of the radioactive atoms. If a radioactive atom, such as ^{33}P is in proximity to an SPA bead, which contains scintillant, electrons can reach the beads and stimulate the scintillant to emit light. However, if a radioactive atom is distant from the beads, the electrons do not reach the bead and no light is emitted. Therefore, radioactive molecules which are in close proximity to the beads, can be distinguished from the molecules which are distant from the beads. SPA technology has been successfully applied for analysis of catalytic action of enzymes, DNA-protein

and protein-protein interactions, receptor binding, radioimmunoassays, uptake of compounds by the cells and their metabolism, screening for DNA binding drugs. However, analysis of RNA-protein interactions or screening for dsRNA binding drugs (VIRIA SPA) is a novel application of SPA.

When streptavidin-SPA beads are mixed with ^{33}P labeled biotinylated dsRNA oligonucleotide, biotin-streptavidin interaction results in binding of the oligonucleotide to the beads. Upon binding, the ^{33}P atom is positioned in close proximity to the beads, and electrons produced during ^{33}P decay reaches the scintillant in the beads. This results in the emission of photons, which can be measured using a scintillation counter. However, the biotin-streptavidin interaction can be sterically prevented if a protein is bound to the dsRNA oligonucleotide prior to its incubation with the beads (biotin protection) (Fig. 9B). When a dsRNA drug capable of binding to dsRNA is introduced into the reaction mixture, the drug containing dsRNA and dsRNA binding protein displaces the dsRNA binding protein from the oligonucleotide. Biotin then becomes available for interaction with streptavidin and emission of photons is restored (Fig. 9C).

SPA can be used not only for the detection of dsRNA binding drugs, but also for establishing whether or not these drugs can bind, and to what extent, any other form of nucleic acids, such as ssRNA, ssDNA or dsDNA, RNA/DNA hybrids, nucleic acids hairpins, bulges, and any form of nucleic acid of any nucleotide sequence, structure, size or conformation.

In one embodiment, a cold nucleic acid competitor, for example DNA, is introduced into the reaction mixture together with the ^{33}P labeled dsRNA. Introducing DNA will not effect the observed signal as compared to a control without DNA, if a dsRNA binding drug does not bind DNA. dsRNA binding proteins, such as E3L or PKR,

do not bind any nucleic acid except long dsRNA. However, the signal will be decreased if a drug can bind both RNA and DNA, especially if DNA is present in excess over RNA.

SPA may also be used to establish if two or more drugs compete for the same or overlapping binding sites on the dsRNA. Data on where drugs bind to the dsRNA can be obtained if a binding site for at least one of the competing drugs is well established.

SPA beads are commercially available from Amersham Pharmacia Biotech and are constructed in such way as to bind specific molecules. Streptavidin coated SPA beads, which can bind biotinylated RNA oligonucleotides are used in one embodiment. However other types of SPA beads, which can bind dsRNA binding protein, such as beads coated with anti-GST antibodies or protein A also could be used in different modifications of the SPA. Binding of the RNA nucleotide to the beads is achieved either directly at the GST moiety of the fusion protein (GST coated SPA beads) or via GST or PKR specific antibodies (protein A coated beads). Non-biotinylated RNA oligonucleotide could be used in these cases.

Biotinylated RNA oligonucleotides are commercially available from Cruachem. Oligonucleotides are preferably close to but not limited to 30 nucleotides in length. Longer and shorter RNA also can be used. Any sequence of oligonucleotides can be used to satisfy specific requirements. For example, for sequence specificity studies all possible 3-nucleotide sequences could be divided between two 34-mer dsRNA oligonucleotides (Fig. 7), and all 4 nucleotide sequences could be divided between ten 30-mer oligonucleotides (Fig. 11). During the synthesis, one or more biotin moieties could be attached anywhere within the oligonucleotide via dU moieties, using spacers of various size. In a preferred embodiment the biotin moiety is attached close to the middle of the 30-

mer nucleotide, for example at position 12, spacer, and hybridized with the complementary oligonucleotide as shown on Fig. 11. Complementary RNA oligonucleotides can be synthesized and hybridized with the biotynilated oligonucleotide. Complementary oligonucleotide or biotinylated oligonucleotide or both can be end-labeled with ^{33}P using gamma ^{33}P ATP (Amersham) and polynucleotide kinase (for example, T4 polynucleotide kinase, New England Biolabs) prior to hybridization. Labeling can be performed under well-known conditions.

DsRNA binding proteins, which have dsRNA binding motifs, for example *Drosophila melanogaster* Staufen, vaccinia virus E3L, and human or mouse PKR can be used in this assay. The dsRNA binding portions of these proteins were expressed as described in Example 1, in E.coli as fusions with glutathione *S*-transferase (GST) (Fig. 1).

Biotin protection experiment

E3L, staufen, mPKR and hPKR were compared in the biotin protection experiment in order to estimate their performance in SPA. Upon binding of a dsRNA binding protein to biotinylated dsRNA, biotin in the RNA is expected to be sterically prevented from binding to streptavidin in the beads (biotin protection). This could be detected by the decrease of the signal emitted from the beads (Fig. 9B). Different proteins are expected to have different biotin protection activity and can exhibit complete or incomplete protection at different protein concentrations.

Biotinylated ^{33}P labeled 30-mer dsRNA oligonucleotide was used in the experiments (Fig.11). Top and bottom strand RNA oligonucleotides (one of them biotinylated) were synthesized at Cruachem, Dulles, VA. ^{33}P end labeling of the RNA oligonucleotides was catalyzed by T4 polynucleotide kinase, which mediates transfer of the ^{33}P phosphate group from ^{33}P gamma ATP to

the 5' end of the RNA oligonucleotide. ^{33}P gamma ATP was produced by Amersham. T4 polynucleotide kinase was available from New England Biolabs, Beverly, MA. Labeling was performed according to the T4 polynucleotide kinase manufacturer's instructions. Top or bottom strand, or both, can be labeled with ^{33}P, however typically only the biotinylated RNA oligonucleotide was labeled. Typically, 2 µl 50,000 nM RNA oligonucleotide (Cruachem), 2.5 µl 10X T4 Polynucleotide kinase buffer (New England Biolabs), 15 µl gamma-^{33}P ATP (10 mCi/ml), 10µl H_2O, 3 µl T4 Polynucleotide kinase (10,000U/ml) were combined in a final volume of 50 µl. The reaction mixture was incubated at 37°C for 30 min and reaction stopped by adding 50 µl 25 mM EDTA. The ^{33}P labeled oligonucleotide was purified from other components of the reaction mixture by gel filtration on the G-25 spin columns (5 Prime-3 Prime, Boulder, CO) according to the manufacturer's instructions. The complementary RNA oligonucleotides were annealed in a 400-500 nM concentration in the annealing buffer (15 mM tris-HCl, pH 7.5, 40 mM NaCl, 2.5 mM EDTA). The mixture was heated to 65-70°C and slowly cooled down to room temperature over a period of 2-5 hours. The produced ^{33}P labeled dsRNA oligonucleotide was diluted to 2 nM concentration, aliquoted and stored at -80° C.

For the biotin protection experiment, reaction mixtures were prepared, which contained 0.25 nM ^{33}P labeled biotinylated dsRNA 30-mer oligonucleotide, 50 mM tris-HCl pH 8.0, 1 mM EDTA, 40 mM NaCl, 0.1% Triton X-100, 5% glycerol, 2 mM ditiothreitol, and 0 to 280 nM of a dsRNA binding protein. The reactions were set up in 96-well Wallac plates, 200 ul/well. The reaction mixtures were incubated for 1.5 hours at room temperature, after which 50 ug/well Streptavidin SPA PVT beads (Amersham) were added and incubation was continued for another 15 min. The plates were sealed and the beads were pelleted to the bottoms of the wells by

centrifugation at 1000g for 3 min. Counting was performed using a Wallac plate reader. hPKR was more active than other proteins in the biotin protection experiment, with nearly complete protection at 0.4-1.2 nM concentration. 0.7 nM hPKR concentration was chosen for further screening experiments. Other proteins were less active, with mPKR requiring 10 nM and E3L 31-93 nM concentration for nearly complete protection. Staufen proved to be the least active of the assessed proteins as even 280 nM concentration of this protein was not sufficient for complete biotin protection (Fig. 12).

As shown in Fig. 12, all four DNA binding proteins were able to prevent interaction of biotynalated dsRNA with streptavidin beads. hPKR had the highest affinity for dsRNA and was the most effective in biotin protection. At a concentration of 0.4 nM, hPKR displaced essentially all dsRNA from interacting with the beads. hPKR was therefore used in high throughout screening of candidate drugs as described in Example 7.

Drugs which are known to bind to dsRNA were next used to demonstrate that drug binding to dsRNA would displace RNA binding proteins. As discussed above, binding of the drug displaces the RNA binding protein and results in an increase in the emission of photons.

The following compounds were screened in this experiment: Bekanamycin sulfate salt (Sigma), Lividomycin A sulfate salt (Sigma), Neomycin sulfate (Sigma), Tobramycin (Fluka), Gentamicin sulfate (Fluka), Netropsin (Sigma), 21x (dimer of netropsin) , Ethidium bromide (Sigma), Acridine orange (Sigma), Distamycin A (Sigma).

Compounds were dissolved in RNA binding buffer (RBB) (50 mM tris-HCl pH 8.0, 1mM EDTA, 40mM NaCl, 0.1% Triton X-100, 5% glycerol 2 mM ditiothreitol). Compounds were added to the reaction mixtures in the 96-well plate, which contained 0.2 nM ^{33}P labeled biotinylated dsRNA oligonucleotide and 0.6 nM of hPKR

in RBB. Serial dilutions of the compounds were made. The resulting volume of the reaction mixtures was 200 µl. Reactions were incubated at room temperature for 90 min, then 10µl streptavidin SPA beads (Amersham) in RBB were added to final concentration 50 µg/well and incubation was continued for another 20 min. The plate was centrifuged at 700 rpm for 2.5 min to sediment the beads, and the scintillation was counted from the bottom of the wells using a Micro Beta Trilux (EG&G Wallac) counter.

The results obtained in SPA corresponded well to the published data on binding of compounds to dsRNA, which were obtained by different methods. Compounds, which are known to strongly bind dsRNA, such as intercalators and neomycin exhibited better binding in SPA than weaker dsRNA binders (Fig. 13). SPA is a reliable method, which can detect even slight differences in binding of compounds to dsRNA. For example, as determined in dsRNA-drug versus dsRNA melting experiments, there is less than 1°C difference in melting temperatures caused by lividomycin (ΔTm=15.2°C) and bekanamycin (ΔTm=14.3°C), indicating that lividomycin is a slightly stronger dsRNA binder than bekanamycin (Biochemistry 36: 11402-11407, 1997). Similarly, lividomycin has a slightly higher affinity than bekanamycin, and as shown in Fig. 13, the drug neomycin (ΔTm=24.7°C)is the strongest binder as shown by SPA. As expected, the DNA binding molecules dystamycin and netropsin exhibited the weakest binding in SPA.

Compounds which bind to both strands of dsDNA or dsRNA are expected to stabilize DNA or RNA duplex by providing additional interaction between the strands. The stronger the compound binds dsRNA or dsDNA the higher temperature is required to melt the duplex. Therefore, binding of compounds to double stranded nucleic acids is routinely measured by thermal denaturation. (Wilson W.D., *et al.*, Biochemistry 32:

4098-4104. 1993; McConnaughie A.W., *et al.*, J. Med. Chem. 37: 1063-1069, 1994; Chen Q., *et al.*, Biochemistry 36: 11402-11407, 1997).

Example 7

High Throughput Screening of a Combinatorial Library to Identify dsRNA-binding Compounds

A library of compounds designed to bind to duplex nucleic acids was designed and synthesized. To date, approximately 200 novel compounds have been synthesized. These compounds were screened in gel band shift assays, SPA, and a fluorescence/quench assay. Twelve of the 144 compounds assayed to-date bound to dsRNA. Table 2 below summarizes the %R values for the twelve hits.

Strongest SPA hits were also positive in gel shifts, weaker SPA hits were apparently below the sensitivity of gel shifts. Fig. 14 shows some of the data obtained through SPA. The x-axis, %R, indicates the relative counts obtained when the compound is present in the assay divided by the counts obtained in the absence of the compound. The %R value indicates a fold increase in photon emission when the compound is present. Therefore, a high value of %R shows that the compound strongly displaces the hPKR from the dsRNA.

Screening of the compounds in SPA was essentially as described in example 6, except that the compounds were dissolved in DMSO to approximate concentration 10 mM. DMSO solutions of the compounds were added to 0.05 mM concentration 200 ul of reaction mixtures containing ^{33}P labeled biotinylated dsRNA 30-mer at 0.2 uM concentration and hPKR at 0.7 nM concentration. Reaction mixtures in the 96-well plates were incubated and processed as described in Example 6. The results were processed using the Microsoft Excel program where %R parameter was calculated (Fig. 14). Compounds which produced a signal statistically greater than the background were considered to be "hits" (Fig. 15). As

presented in Fig. 14, 9 hits, which ranged from 8 to 170 %R, are shown. The %R values for all twelve hits were confidently above the background. Even for the weakest hits for series 1a with %R as low as T-test values were statistically significant: A5 - 0.006, C10-0.0002.

The compounds were also screened in gel shifts, which were performed essentially as described in Example 3, except hPKR at 0.7 nM concentration was used instead of E3L. Screening was performed in duplicates at 2 concentrations of the compounds -- 1mM and 0.2 mM. 3 compounds, B4, B5 and B6, which were the strongest hits in SPA, were also detected in gel shifts. Compounds B4 and B5 were comparable in activity to a strong dsRNA binding antibiotic, neomycin, whereas compound B6 was considerably weaker. Gel shift data correlate well with the SPA data, where compound B6 was also a much weaker hit than compounds B4 and B5.

In order to investigate dsRNA structure specificity of binding of series 1a compounds, B4, B5 and B6 were also characterized by direct monitoring of binding to dsRNA and ssRNA. 1 mM of each compound was incubated for 1 hour at room temperature with 0.2 nM dsRNA (24-mer) or ssRNA (30-mer). The reaction mixtures were loaded onto 6% RNA retardation gels and were processed using a Storm phosphoimager. All three compounds bound to dsRNA as observed by a shift in the mobility of the dsRNA. There was no significant shift in ssRNA by B5 and B6, although direct binding of compound B4 to ssRNA was observed, indicating specificity for dsRNA.

TABLE 2

COMPOUND	%R
Series 1a	
A5/GEN00697	8
A9/GEN00699	9
A11/GEN0066	10
B4/GEN0067	170
B5/GEN00700	140
B6/GEN0056	31
B7/GEN0018	8
C2/GEN0048	17
C10/GEN0077	8
D12/GEN0063	9
Series 1b	
B11/GEN00120	9
D8/GEN00158	22

$$\%R = \frac{\text{dsRNA·compound - dsRNA·hPKR}}{\text{dsRNA - dsRNA·hPKR}}$$

Example 8

Fluorescence/Quench Assay

An assay based on fluorescence resonance energy transfer (FRET) has been developed to identify dsRNA-binding compounds. FRET is a phenomenon in which fluorescent photons are detectably transferred from one fluorescent molecule to another. In most FRET reactions, the first dye is excited at a given wavelength of light (the excitation maxima). The light emitted from the first dye is efficiently transferred to

the second dye, usually because of significant overlap between the emission spectra of the first dye and the excitation spectra of the second. The second dye absorbs the photon given off from the first dye and emits a longer wavelength, lower energy fluorescent photon.

Another type of commonly used FRET pair is the donor/quencher dye pair. In this interaction, the resonance energy transferred from the first to the second dye is not given off as a second light emission, but instead is thermally dissipated into the system. Therefore, the close proximity of the "quenching" dye to the fluorescent dye causes a significant decrease in fluorescent light given off by the system. Dabcyl is a label that has become increasing popular as a sort of "universal" quenching moiety, because it effectively quenches the fluorescence of many different fluorophores.

The binding of ligands to double-stranded nucleic acids stabilizes the duplex, or helical, form of the DNA or RNA. In the past, thermal melting experiments were used to show that the binding of almost all ligands to DNA or RNA causes an increase in the Tm. (In very few known cases, ligands have been found that bind to, and therefore, stabilize single-stranded DNA.) In the early fifties it was shown that salt caused the Tm of DNA to increase. Since then, it has been shown that the binding of polyamines, small-molecule drugs, peptides, and proteins all cause an increase in Tm upon binding to nucleic acids.

The FRET assay utilizes short, end-labeled, complementary, "indicator oligonucleotides" which are single-stranded under standard assay conditions. Ligand binding drives the formation of a stable duplex structure in some of these indicator oligonucleotides. In the duplex conformation, the quenching dye, Dabcyl, is brought into close proximity to a fluorescent dye

(fluorescein, or the like) in these "indicator oligonucleotides" causing a sharp decrease in fluorescence. Thus, binding of the ligand to that particular "indicator oligo" causes a decrease in fluorescence.

This assay is the subject of a co-pending application USSN _____, assigned to the present assignee. This co-owned application (USSN _____) in its entirety is herein incorporated by reference.

This assay was used in conjunction with gel SPA and bandshift assays to screen the structure biased chemical library. Specificity of B5 binding to dsRNA was also corroborated in a fluorescence quenching competition assay. In the competition assay, non-labeled dsRNA, or ssRNA is added to the assay mixture at increasing concentrations. If drug is able to bind any of these non-labeled oligos, the quenching effect which is observed after binding of the drug to the F/Q-RNA is reduced. Lividomycin, known to bind specifically to dsRNA but not dsDNA, was used to test the new assay format; dsRNA and dsDNA were used as competitors. Only dsRNA was able to bring the fluorescence signal to the original levels, indicating that this is a specific competition assay.

Next, fluorescence/quenching competition experiments were performed with Series 1a B5 compound in order to investigate preference of its binding to different forms of nucleic acids. In these experiments, B5 bound to fluorescein/dabcyl labeled dsRNA successfully competed with dsDNA, ssRNA and DNA/RNA hybrid. For compound B5, indicating that compound B5 preferentially binds to dsRNA.

The following oligonucleotides were used in the assay: 24mer 24DB, 5'-d(TTATTCTGTCGTG CTGGTTTATTC); 24mer 24DT, 5'-d(GAATAAACCAGCACGACAGAATAA); 24mer 24J, 5'-r(GAAUAAACCAGCACGACAGAAUAA); 24mer 24JB, 5'-r(UUAUUCUGUCGUGCUGGUUUAUUC); 14mer Vir51, 5'-r(FCUAGAUC

UGAACUU) and 9mer Vir55, 5'-r(CAGAUCUAGQ), F denotes fluorescein and Q dabcyl.

dsRNA (24J+24JB), dsDNA (24DT+24DB) and DNA/RNA hybrid (24JB+24DT) were formed by mixing the respective oligonucleotides at 100μM final concentration in 10 mM HEPES pH7.5, 1 mM EDTA and 10 mM NaCl, heating the mixtures to 80°C for 5 min and allowing them to slowly cool to room temperature for 3 h.

Direct monitoring of drug binding was performed by adding 25 nM Vir51 and 40 nM Vir55 to the respective drug to a total volume of 200 ul. The drug concentration used are indicated in the figure legends. For the competition experiments the competitor nucleic acid dsRNA, dsDNA, DNA/RNA hybrid and ssRNA (24JB) was added to Vir51/Vir55 mixture at two different concentrations (0.5 and 1 μM) and immediately added to the respective concentration of the drug and the relative fluorescence measured. All experiments were performed in a buffer solution containing 10 mM HEPES pH7.5, 1 mM EDTA and 10 mM NaCl. Measurements were performed immediately after mixture using Cytofluor Fluorescence Reader, Multi-well Plate Reader Series 4000 (Perseptive Biosystems). Excitation was 485 with bandwidth 20 and emission 530 with bandwidth 25 and a gain of 70. At 10μM B5, only dsRNA, but not dsDNA, ssRNA or DNA/RNA hybrid was able to bring the signal quenched by B5 to the original level, indicating preference of B5 binding to the dsRNA over dsDNA, ssRNA and DNA/RNA hybrid (see Fig. 15A-D). These results are consistent with the results obtained in the SPA and gel shift assays).

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

1. A method of screening for a compound capable of altering base pair sequence-independent binding of a dsRNA-binding protein or protein complex to a dsRNA fragment, comprising the steps of:

(a) forming a reaction mixture composed of (i) said dsRNA-binding protein or protein complex, and (ii) a duplex dsRNA fragment to which said binding protein bind, independent of dsRNA base pair sequence;

(b) detecting the level of binding of said dsRNA-binding protein to said dsRNA fragment in said dsRNA fragment in the presence and absence of said compound;

(c) comparing the binding observed in step (b);

(d) selecting the compound if the difference in binding observed in the presence and absence of the compound is greater than a selected value.

2. The method of claim 1, wherein said compound binds to dsRNA with at least about 2-3 fold higher affinity than to a polynucleotide selected from the group consisting of single-stranded RNA, single-stranded DNA, and double-stranded DNA.

3. The method of claim 1, wherein the concentration of said RNA-binding protein or protein complex is present in an amount effective to form a complex with at least 50% of the dsRNA, in the absence of said compound.

4. The method of claim 1, wherein the duplex dsRNA fragment is between about 10-100 base pairs in length.

5. The method of claim 4, wherein the duplex dsRNA fragment is between about 16-50 base pairs in length.

6. The method of claim 5, wherein the duplex dsRNA fragment is between about 30-50 base pairs in length.

7. The method of claim 1, for use in identifying candidate anti-viral compounds, wherein the duplex dsRNA-binding protein is a viral protein.

8. The method of claim 7, wherein the viral protein is selected from the group consisting of E3L, sigma3, E3L dsRNA-binding domain and sigma3 dsRNA-binding domain.

9. The method of claim 8 wherein the viral protein is E3L or the dsRNA-binding domain thereof.

10. The method of claim 1, wherein the duplex dsRNA-binding protein is a cellular protein selected from the group consisting of PKR, DRAF1, DRAF2, RNAaseH, Z-DNA binding protein, La (SS-B), TRBP, and dsRNA-specific adenosine deaminase.

11. The method of claim 10, wherein said binding protein is PKR.

12. The method of claim 1, wherein the binding of the RNA-binding protein or protein complex to dsRNA is detected by a gel band shift assay.

13. The method of claim 1, wherein the binding of the RNA-binding protein or protein complex to dsRNA is detected by a scintillation proximity assay.

14. The method of claim 1, wherein the binding of the RNA-binding protein or protein complex to dsRNA is detected by a filter binding assay.

15. The method of claim 1, wherein the binding of said compound to dsRNA is base pair sequence-independent.

16. A method of treating a subject infected with an RNA virus comprising administering to the subject a pharmaceutically effective amount of a compound selected by:

(a) forming a reaction mixture composed of (i) said dsRNA-binding protein or protein complex, and (ii) a duplex dsRNA fragment to which said binding protein bind, independent of dsRNA base pair sequence;

(b) detecting the level of binding of said dsRNA-binding protein to said dsRNA fragment in said dsRNA fragment in the presence and absence of said compound;

(c) comparing the binding observed in step (b);

(d) selecting the compound if the difference in binding observed in the presence and absence of the compound is greater than a selected value.

17. The method of claim 16 wherein said compound binds to dsRNA with at least 2-3 fold higher affinity than to a polynucleotide selected from the group consisting of single-stranded RNA, single-stranded DNA, or double-stranded DNA.

18. The method of claim 16, wherein said compound binds to about 10-100 base pairs of said duplex dsRNA.

19. The method of claim 16, wherein said compound binds to about 16-50 base pairs of said duplex dsRNA.

20. The method of claim 16, wherein said compound binds to about 30-50 base pairs of said duplex dsRNA.

21. The method of claim 16, wherein the binding of said compound to dsRNA is base pair sequence-independent.

22. The method of claim 16, wherein said compound comprises a concatemer of at least two subunits, each of which is selected by:

(a) forming a reaction mixture composed of (i) said dsRNA-binding protein or protein complex, and (ii) a duplex dsRNA fragment to which said binding protein bind, independent of dsRNA base pair sequence;

(b) detecting the level of binding of said dsRNA-binding protein to said dsRNA fragment in said dsRNA fragment in the presence and absence of said compound;

(c) comparing the binding observed in step (b);

(d) selecting the compound if the difference in binding observed in the presence and absence of the compound is greater than a selected value.

23. A multimeric dsRNA-binding agent comprising a linear concatemer formed by covalently linking at least a first and a second dsRNA-binding compound, each of which is selected by steps (a)-(d) of claim 1.

24. The dsRNA-binding agent of claim 23, wherein the binding of at least one of said dsRNA-binding compounds to dsRNA is base pair sequence-independent.

25. The dsRNA-binding agent of claim 23, wherein the binding of each of said dsRNA-binding compounds to dsRNA is base pair sequence-independent.

26. The RNA-binding agent of claim 23, which further includes an RNA modifying agent or an RNA cleaving agent covalently linked to one of the dsRNA-binding compounds.

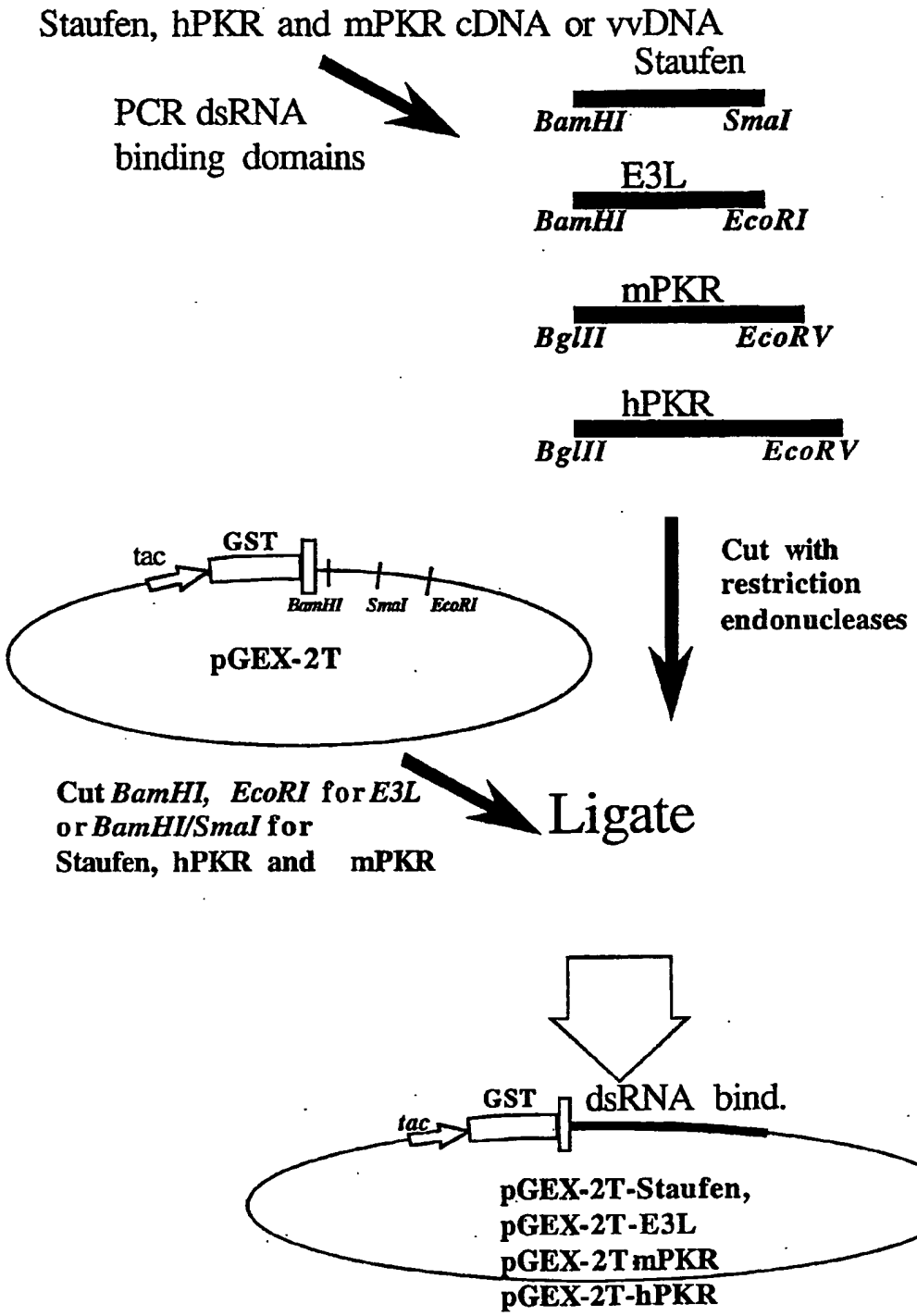
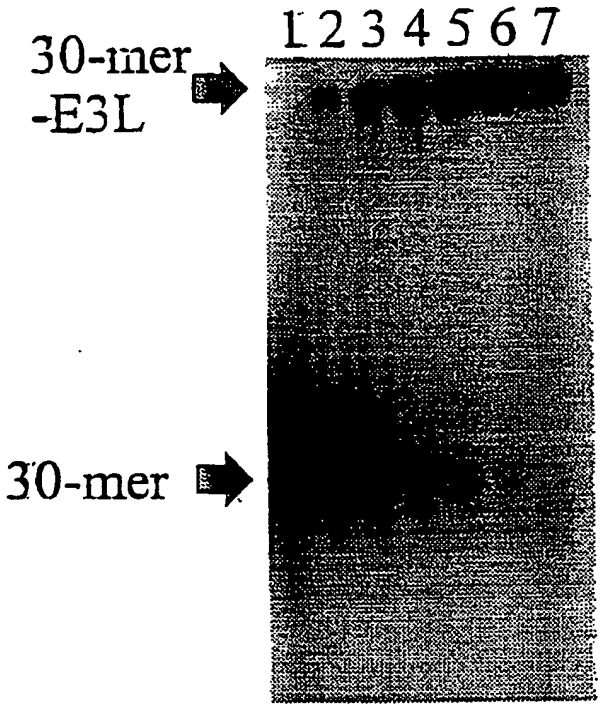

Staufen, hPKR and mPKR cDNA or vvDNA
PCR dsRNA binding domains
Staufen
BamHI SmaI
E3L
BamHI EcoRI
mPKR
BglII EcoRV
hPKR
BglII EcoRV
tac
GST
BamHI SmaI EcoRI
pGEX-2T
Cut with restriction endonucleases
Cut BamHI, EcoRI for E3L or BamHI/SmaI for Staufen, hPKR and mPKR
Ligate
tac
GST
dsRNA bind.
pGEX-2T-Staufen,
pGEX-2T-E3L
pGEX-2T mPKR
pGEX-2T-hPKR

Fig. 1

Fig. 2



1-no E3L, 2-1nM, 3-4nM, 4-12nM, 5-40nM, 6-120nM, 7-400nM E3L

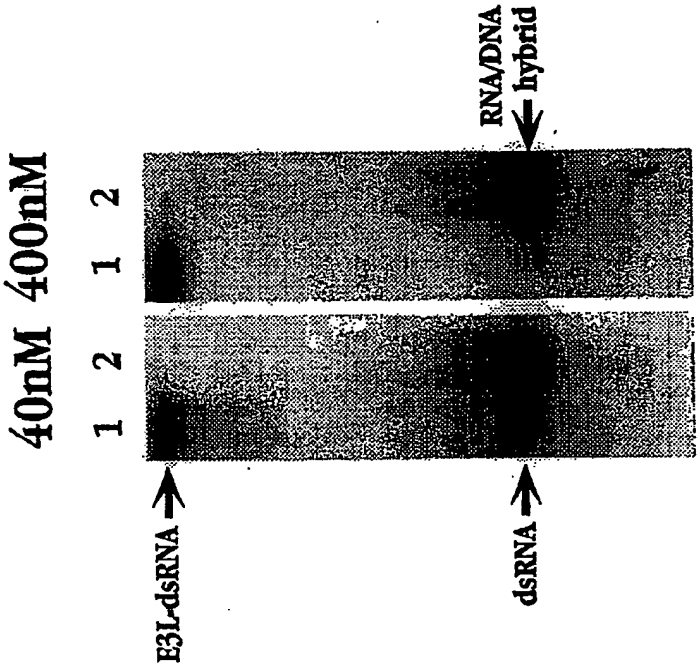

RNA/DNA hybrid
40nM 400nM
1 2 1 2
E3L-dsRNA
dsRNA

Fig. 3B

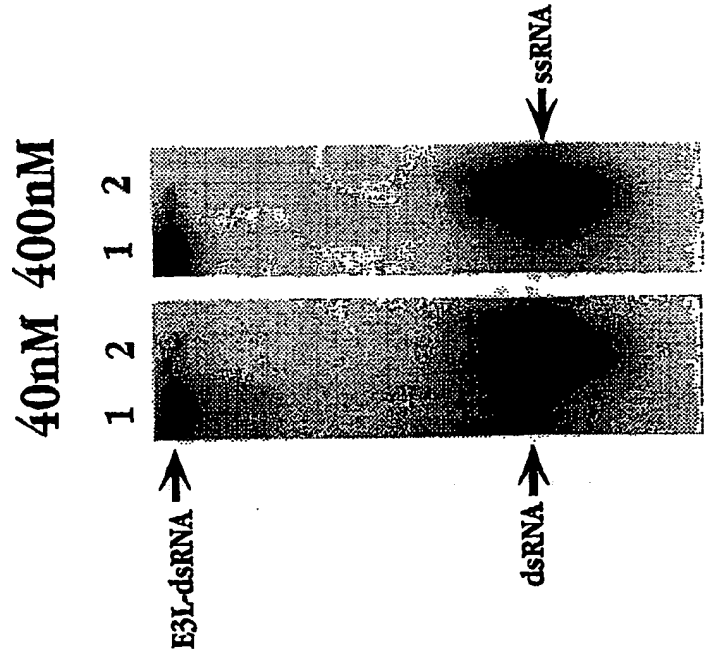

ssRNA
40nM 400nM
1 2 1 2
E3L-dsRNA
dsRNA

Fig. 3A

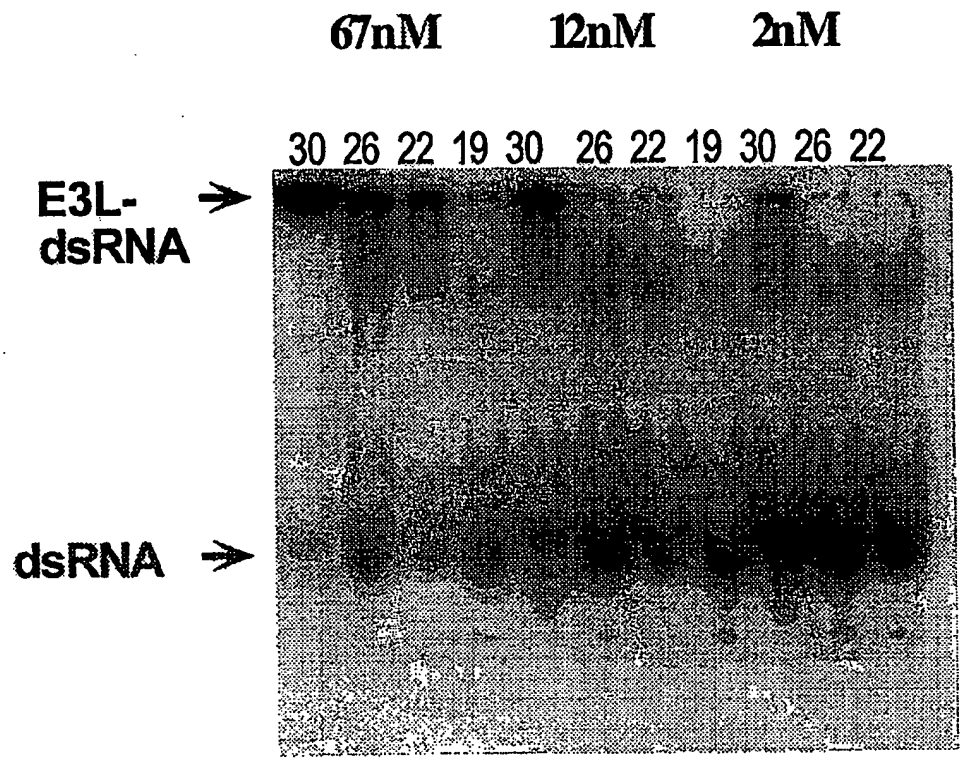


Fig. 4

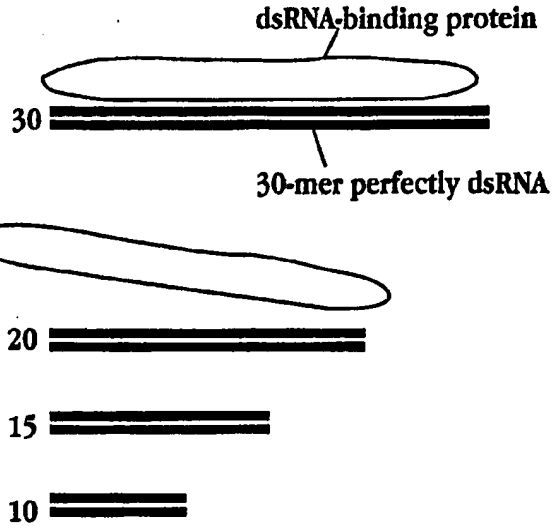


Fig. 5A

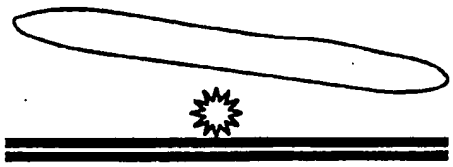


Fig. 5B

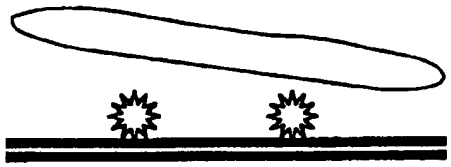


Fig. 5C

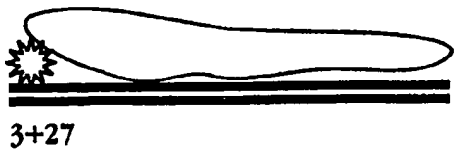
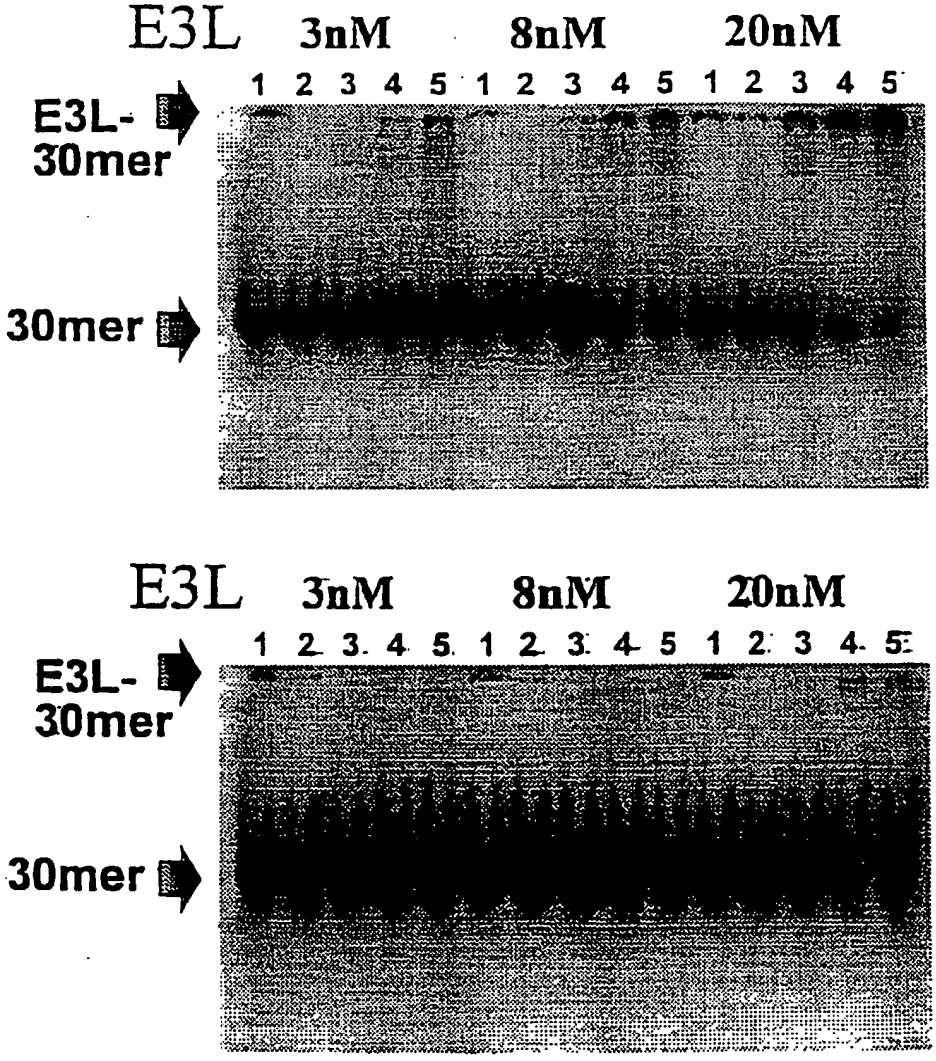


Fig. 5D

Fig. 6A



A: gel w/o EtBr, B: Gel with 3.3 μM EtBr.
EtBr concentrations: 1-90μM
2-30μM
3-10μM
4-3.3μM
5-0

Fig. 6B

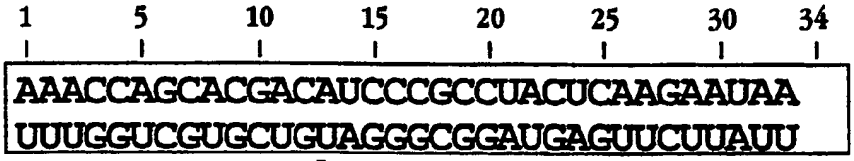


Fig. 7A

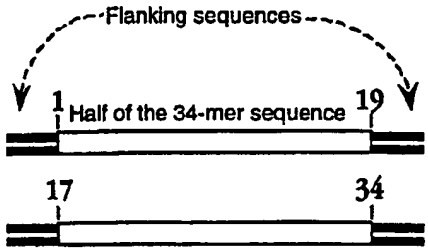


Fig. 7B

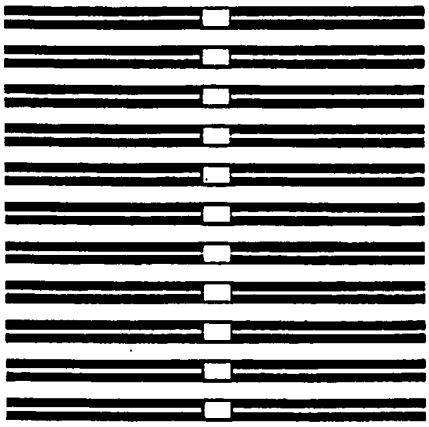


Fig. 7D

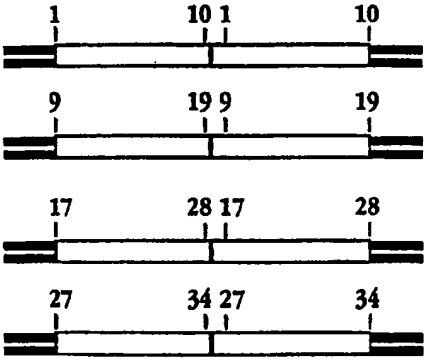


Fig. 7C

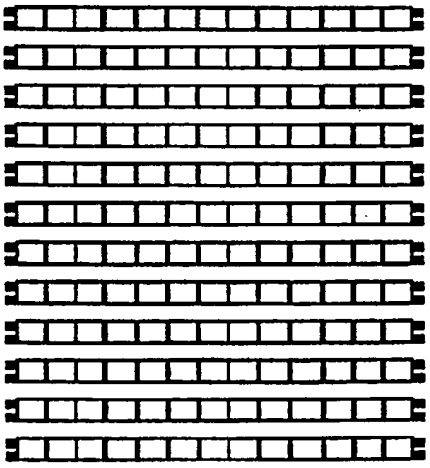


Fig. 7E

ACGACAGAAUAAACCAGCACGACAGAAUAA SEQ ID NO: 19

Fig. 7F

UACUCAUCCCGCCUACUCAAGGGAUGAUCC SEQ ID NO: 20

Fig. 7G

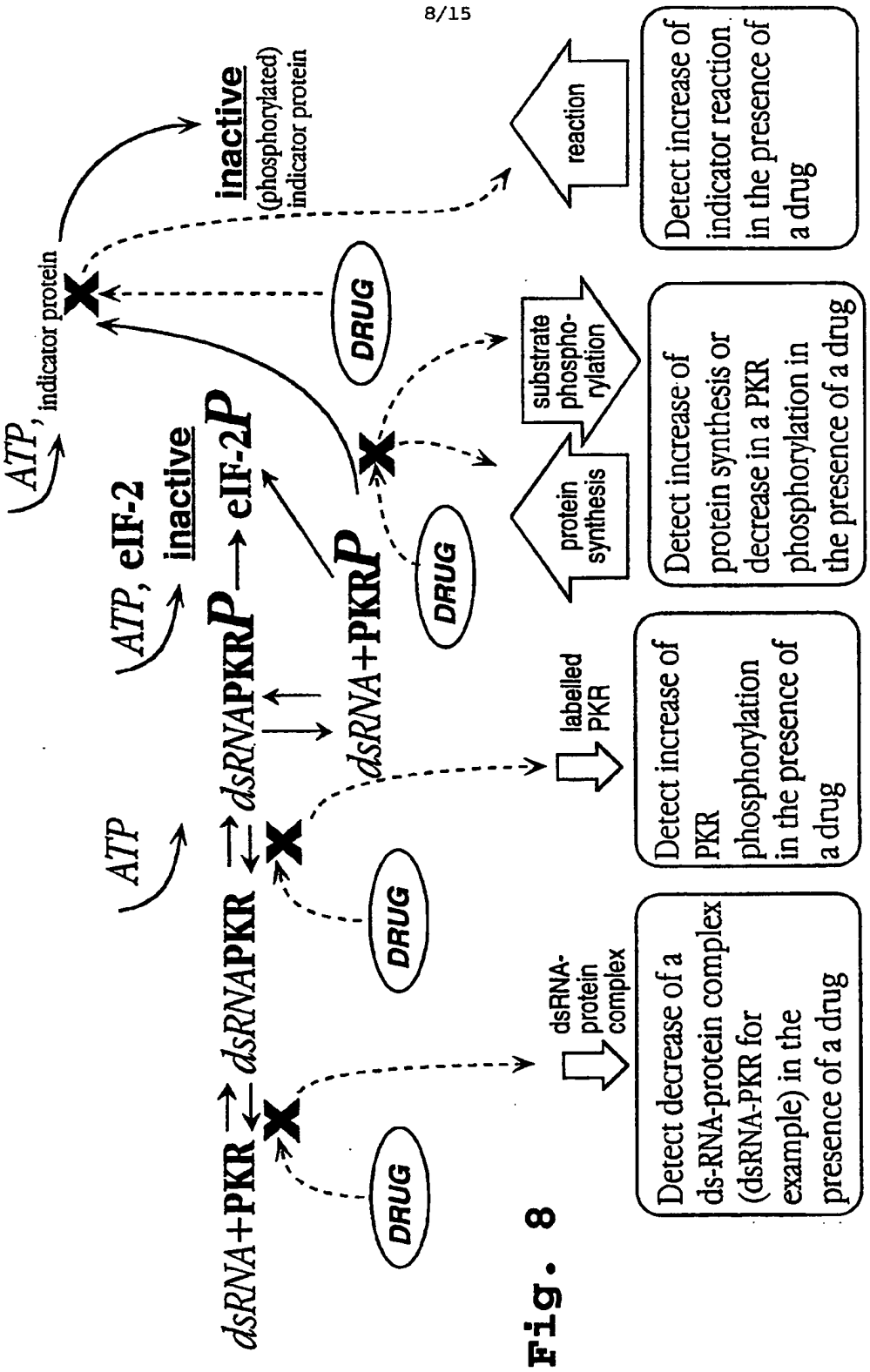

dsRNA+PKR ⇄ dsRNAPKR ⇄ dsRNAPKRP → eIF-2P
ATP
ATP, eIF-2
inactive
ATP, indicator protein
inactive
(phosphorylated) indicator protein
dsRNA+PKRP
DRUG
DRUG
DRUG
DRUG
dsRNA-protein complex
labelled PKR
protein synthesis
substrate phospho-rylation
reaction
Detect decrease of a ds-RNA-protein complex (dsRNA-PKR for example) in the presence of a drug
Detect increase of PKR phosphorylation in the presence of a drug
Detect increase of protein synthesis or decrease in a PKR phosphorylation in the presence of a drug
Detect increase of indicator reaction in the presence of a drug

Fig. 8

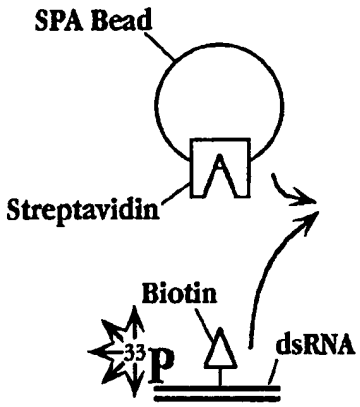


Fig. 9A

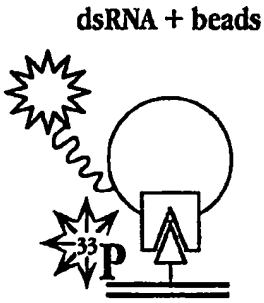


Fig. 9B

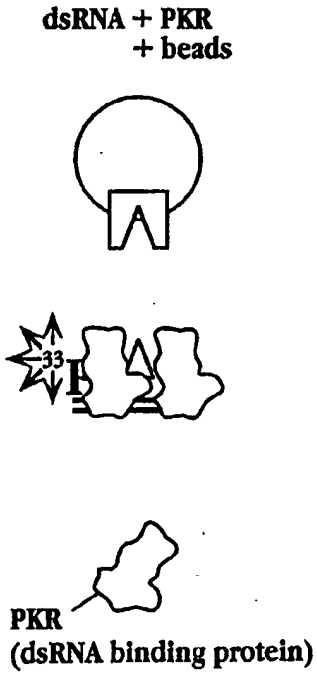


Fig. 9C

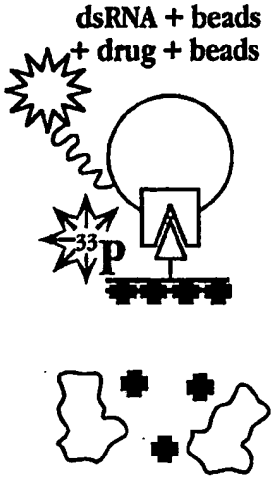


Fig. 9D

	SEQ. ID NO:
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CGUACUGCAAAAGGAUCCGUACUGCAAAAG	23
UGACGUUUAAUACAAGCUGACGUUUAAUAC	24
AGAUAAUUCAACACUAcAGAUAAUUCAACA	25
AUAGGCGCGACAAACAUAUAGGCGCGACAA	26
AAAUCAUUGGGUACAAGAAAUCAUUGGGUA	27
GAACCCUCCACACUCGGGAACCCUCCACAC	28
AGGACCUUAGAGACACCAGGACCUUAGAGA	29
UCACGCACUUCGUAGACUCACGCACUUCGU	30

Fig. 10

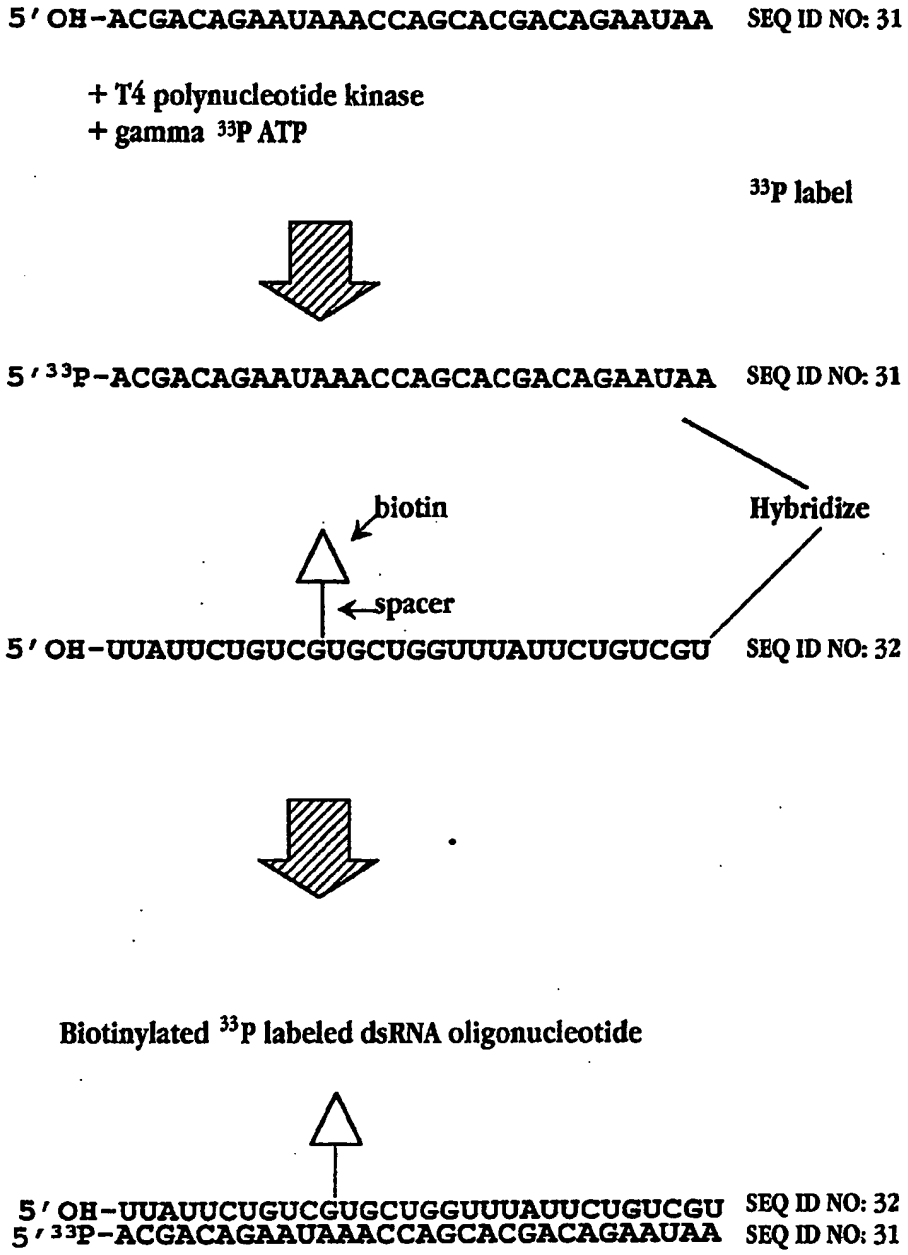

5′ OH-ACGACAGAAUAAACCAGCACGACAGAAUAA SEQ ID NO: 31
+ T4 polynucleotide kinase
+ gamma 33P ATP
33P label
5′ 33P-ACGACAGAAUAAACCAGCACGACAGAAUAA SEQ ID NO: 31
biotin
Hybridize
spacer
5′ OH-UUAUUCUGUCGUGCUGGUUUAUUCUGUCGU SEQ ID NO: 32
Biotinylated 33P labeled dsRNA oligonucleotide
5′ OH-UUAUUCUGUCGUGCUGGUUUAUUCUGUCGU SEQ ID NO: 32
5′ 33P-ACGACAGAAUAAACCAGCACGACAGAAUAA SEQ ID NO: 31

Fig. 11

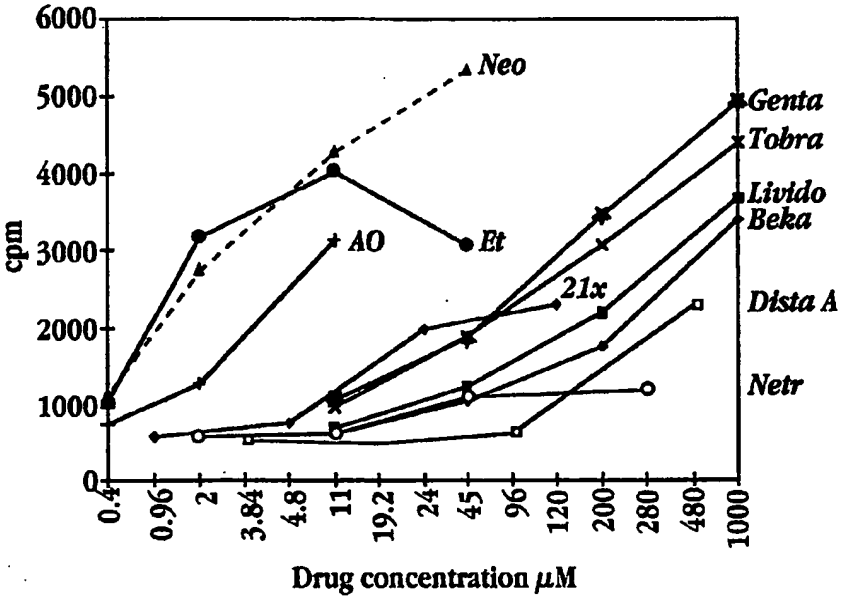

6000
5000
4000
3000
2000
1000
0
cpm
Neo
Genta
Tobra
Livido
Beka
Dista A
Netr
AO
Et
21x
0.4
0.96
2
3.84
4.8
11
19.2
24
45
96
120
200
280
480
1000
Drug concentration μM

Fig. 13

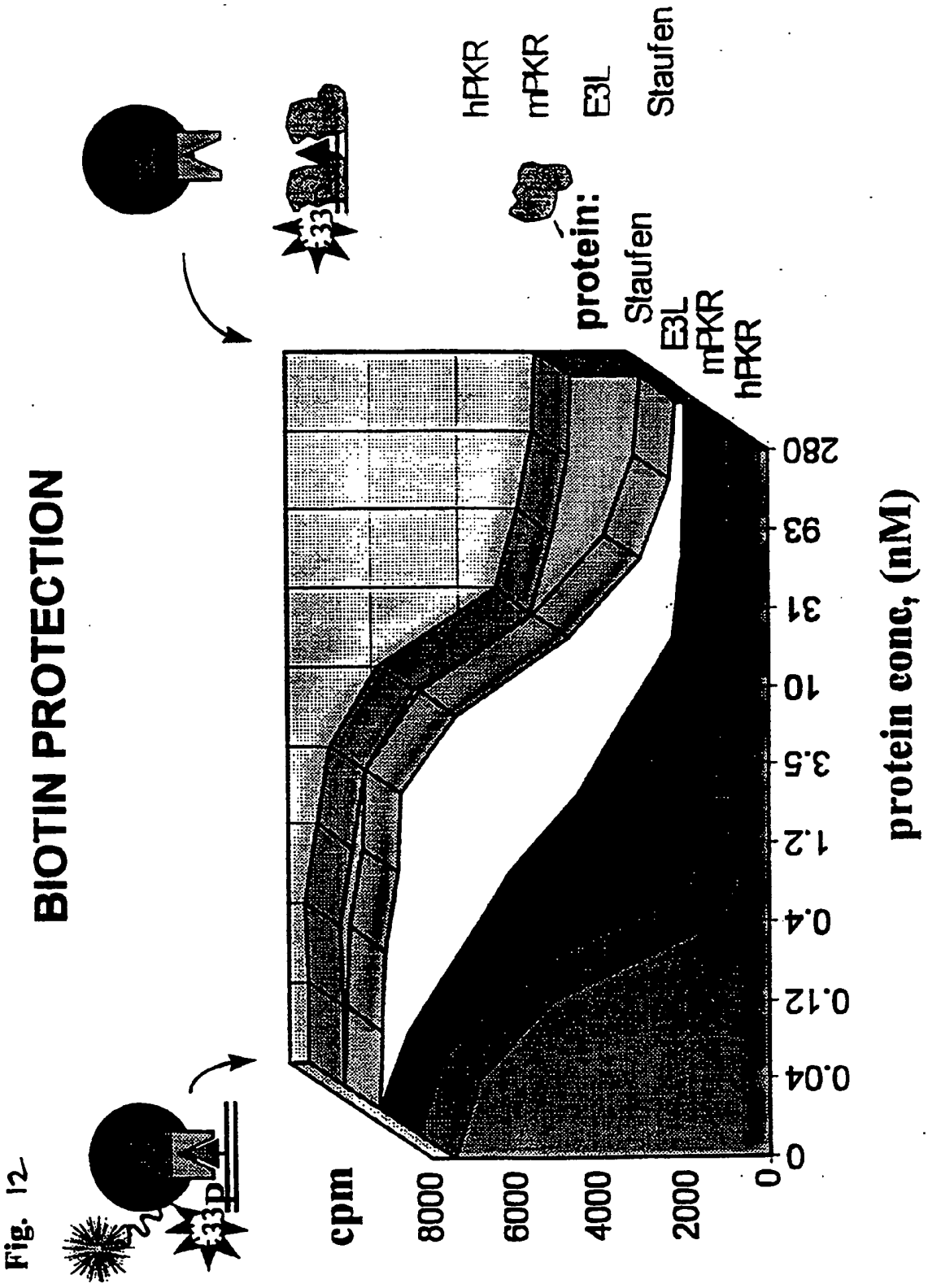
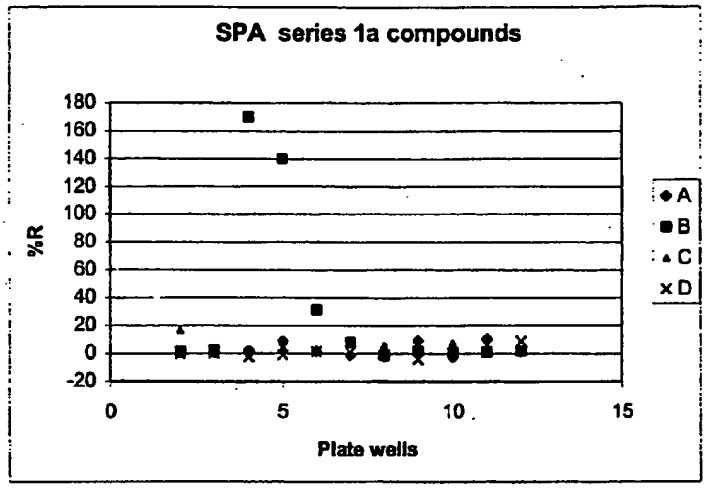
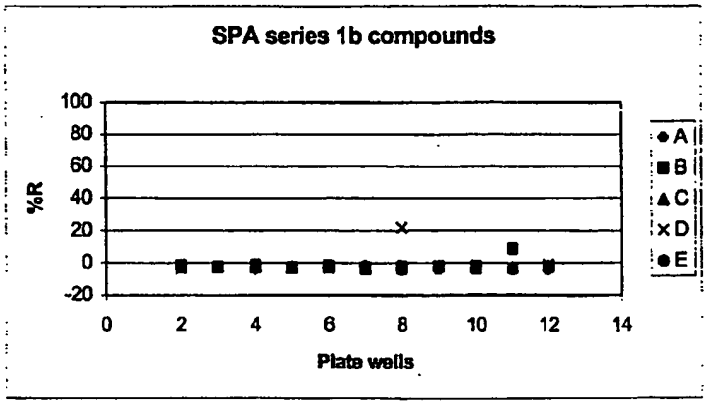

BIOTIN PROTECTION
cpm
8000
6000
4000
2000
0
protein conc, (nM)
280
93
31
10
3.5
1.2
0.4
0.12
0.04
0
protein:
hPKR
mPKR
E3L
Staufen
hPKR
mPKR
E3L
Staufen
33P
33P

Fig. 12

Fig. 14


SPA series 1a compounds
%R
180
160
140
120
100
80
60
40
20
0
-20
0
5
10
15
Plate wells
A
B
C
D


SPA series 1b compounds
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80
60
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2
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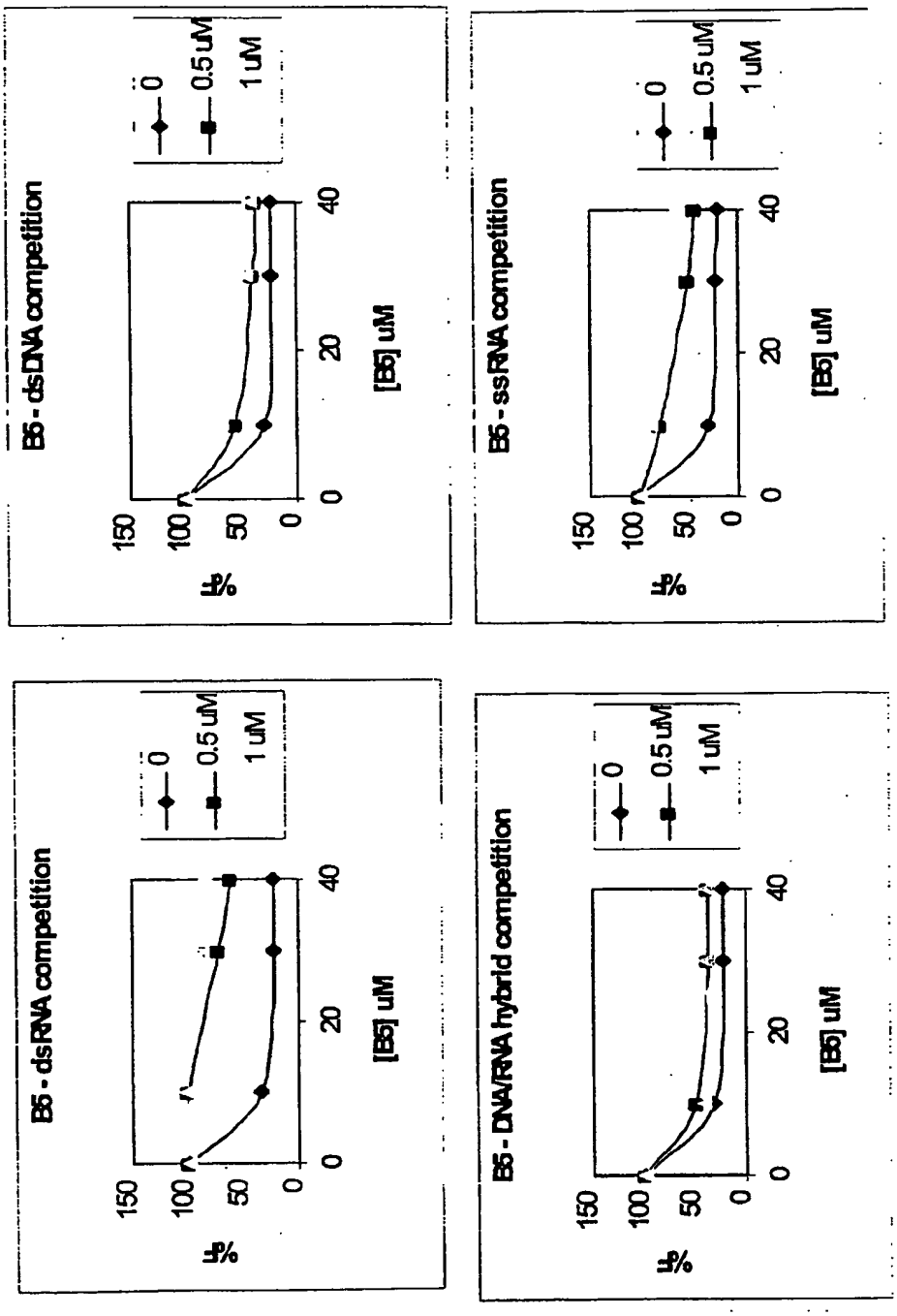
6
8
10
12
14
Plate wells
A
B
C
D
E

Fig. 15


B5 - dsDNA competition
B5 - ssRNA competition
B5 - dsRNA competition
B5 - DNA/RNA hybrid competition
0
0.5 uM
1 uM
[B5] uM
%F
150
100
50
0
0
20
40

SEQUENCE LISTING

<110> Alexander S. Belyaev
Thomas Wayne Bruice
Cynthia A. Edwards
Kirk E. Fry
Lisa M. Turin

<120> dsRNA/dsRNA-Binding Protein Methods and Compostition

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<210> 26
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<223> synthesized oligonucleotide

<400> 26
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<213> Artificial Sequence

<220>
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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/19203

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C12Q1/68 C07K14/00 A61K38/16 //C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 00446 A (GENELABS INC) 7 January 1993 see the whole document	1-26
X	WO 94 14980 A (GENELABS TECH INC) 7 July 1994 see the whole document	1-26
A	WO 96 40948 A (RES CORP TECHNOLOGIES INC) 19 December 1996 see the whole document	1-26
A	WO 94 17087 A (UNIV CALIFORNIA) 4 August 1994 see claims 18-21	1-26

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 1999	16/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/19203

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BENKIRANE M ET AL: "ONCOGENIC POTENTIAL OF TAR RNA BINDING PROTEIN TRBP AND ITS REGULATORY INTERACTION WITH RNA-DEPENDENT PROTEIN KINASE PKR" EMBO JOURNAL, vol. 16, no. 3, 3 February 1997, pages 611-624, XP002073123 see the whole document	1-26
A	WO 97 00949 A (MASSACHUSETTS INST TECHNOLOGY) 9 January 1997 see the whole document	1-26
A	BEVILACQUA P ET AL: "Minor groove recognition of double stranded RNA by the double stranded RNA binding domain from the RNA activated protein kinase PKR" BIOCHEMISTRY, vol. 35, 1996, pages 9983-94, XP002091877 cited in the application see the whole document	1-26
X	WO 95 11922 A (AFFYMAX TECH NV ;DOWER WILLIAM J (US); MATTHEAKIS LARRY C (US)) 4 May 1995 see page 34	23-25
A	WO 94 21825 A (ISIS PHARMACEUTICALS INC) 29 September 1994 see the whole document	1

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/19203

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 16-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/19203

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